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THE ROLE OF A NEWLY DISCOVERED EXOTOXIN (S TOXIN) IN 'PSEUDOMON--ETC(U)
AUG 78 B H IGLEWSKI, M R THOMPSON DAMD17-78-C-8020

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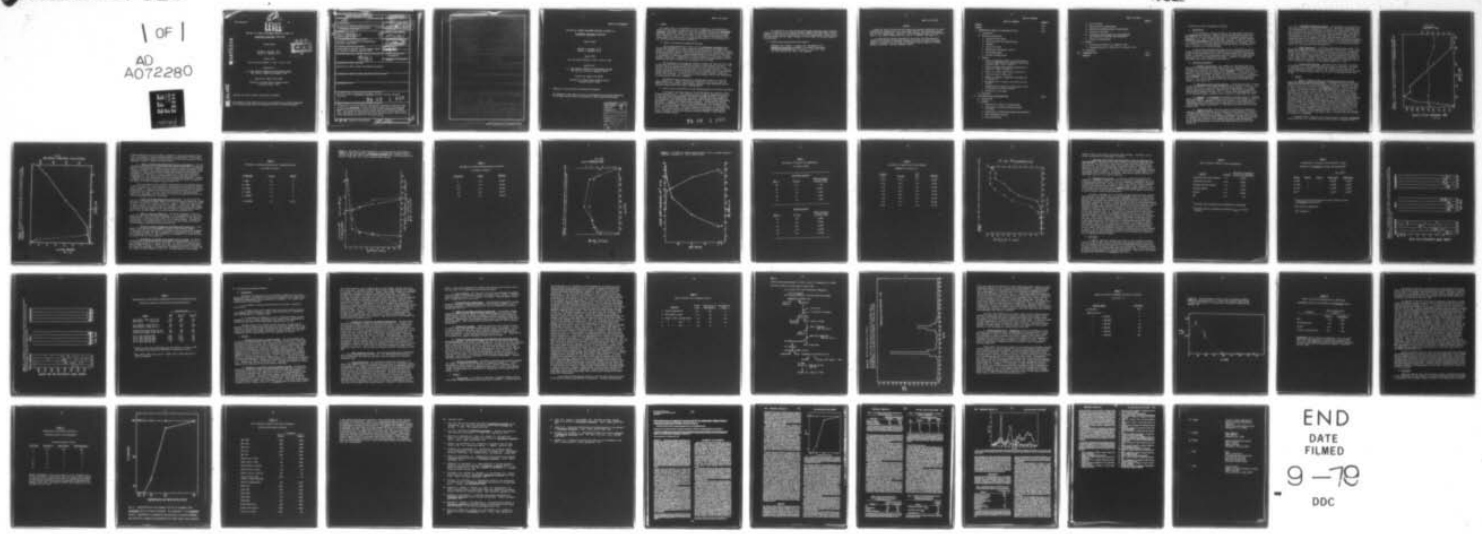

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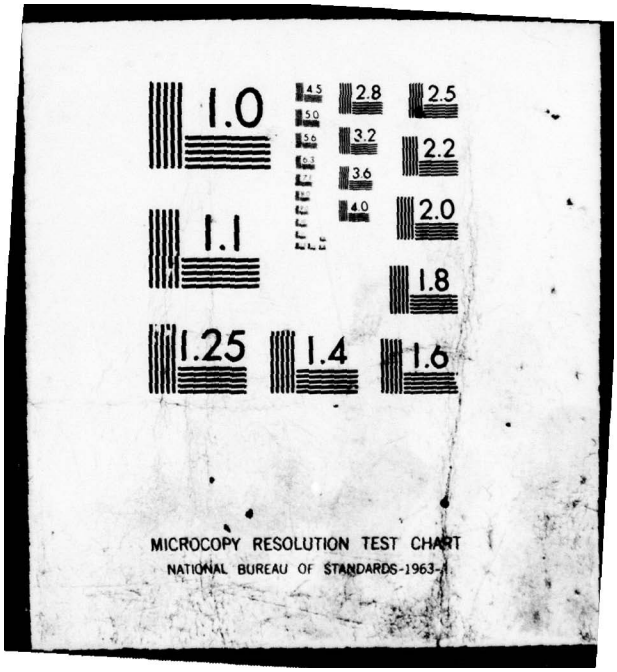
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FINAL REVISION

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LEVEL II

THE ROLE OF A NEWLY DISCOVERED EXOTOXIN (S TOXIN) IN PSEUDOMONAS AERUGINOSA INFECTIONS

Annual Report

Barbara H. Iglewski, Ph.D.
Michael R. Thompson, Ph.D.

August 1978

For the period February 1, 1978 - July 31, 1978

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

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University of Oregon Health Sciences Center
Portland, Oregon 97201

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Exoenzyme S is an extracellular ADP-ribosyl transferase produced by some strains of P. aeruginosa. We have studied the production of S in vivo and in vitro and have partially purified S from culture supernatants of strain #388. This enzyme has been shown to differ from toxin A in 1) size, 2) substrate specificity, 3) thermal stability, 4) enzyme activation, and 5) nutritional requirements necessary for its production.

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D. SUMMARY

Our overall goal is to determine the role of exotoxin S (an extracellular enzyme with ADP-ribosyl transferase activity) in humans infected with Pseudomonas aeruginosa and develop vaccines to reduce the morbidity and mortality associated with these infections. During the period of the project covered in this Annual Report (February 1, 1978 - July 31, 1978) two main lines of investigation have been pursued: (a) Production of S in vitro and in vivo and (b) Purification of S and characterization of partially purified S.

A. Study of S production in vitro and in vivo.

The in vitro production of toxin S (S) by Pseudomonas aeruginosa strain 388 (Ps 388) was studied in order to maximize yields of S for its purification. Yields of S depended on the concentration of nitrilotriacetic acid (NTA), iron and monosodium glutamate (MSG) in the culture medium and were not significantly affected by the presence of glycerol. Various complex media were compared and a dialysate of tripticase soy broth supplemented with NTA and MSG, pH 7.5 was found to support maximum S production. Production of S required aeration and was greatest at 32° C. Production was also correlated with the growth cycle of Ps 388.

The production of S in vivo was studied in burned mice infected with Ps 388 by measuring the ADP-ribosyl transferase activity in skin extracts and serum. Significant levels of enzyme activity were detected in skin extracts as early as 18 hrs. post infection and in serum as early as 24 hrs. post infection. S activity in serum continued to increase through 48 hrs. post infection (all Ps 388 infected animals died by 52 hrs). S activity in the skin extracts reached a plateau at 24 hrs. post infection. The S specificity of this enzyme activity was demonstrated by neutralization with anti S antibody.

Preliminary results suggest that A antibody may protect mice from the lethal effects of S. However, we have also found anti S activity in some pre-immunized serum from several animal species, therefore these experiments must be repeated using purified anti toxin A immunoglobulin.

B. Studies on the purification of S and characterization of partially purified S.

A proteolytic deficient mutant of Ps 388 (Ps 388-6) was isolated to facilitate purification of S. S was partially purified from 16L fermenter batches of Ps 388-6 employing batch adsorption and elution from DEAE-cellulose followed by ion exchange column chromatography, isoelectric focusing and polyacrylamide gel electrophoresis. The partially purified S was used to raise S enzyme neutralizing antibodies in rabbits and to begin characterizing S. By assaying the specific ADP-ribosyl transferase activity of S we established that S is relatively heat stable, is not potentiated by urea and DTT (as is toxin A) but is stabilized with DTT and potentiated by SDS. The latter suggests the existence of a proenzyme form of S. The S has an isoelectric point of 4.5 and on SDS-polyacrylamide gels 2 peaks of enzymatic activity are found with molecular weights of approximately 60,000 and 30,000 daltons.

The feasibility of using immunochromatography employing toxin A antibody coupled to sepharose 4B was further explored. However less than 5-10% of the S present in crude culture supernatants or in DE-52 eluates bound to those columns. Thus this method does not appear feasible to use at least in the early stages of S purification.

C. Publications resulting from this research.

Iglewski, B.H., Sadoff, J., Bjorn, M.J. and Maxwell, E.S. Pseudomonas aeruginosa exoenzyme S: An adenosine diphosphate ribosyltransferase distinct from toxin A. Proc. Nat. Acad. Sci: USA 75,3211-3215, 1978. Copy appended.

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FORWARD

During the course of this work the authors were greatly assisted by Mr. David Oldenburg, Mr. Michael Bjorn, Mr. Dennis Ohman and Mrs. Joan Rittenberg, their help is deeply appreciated. Portions of this research were done in collaboration with Dr. J.C. Sadoff, WRAIR, Wash. D.C. and Dr. E. Maxwell, NIH, Wash. D.C.

In conducting the research described in this report the investigator(s) adhered to the "Guide for Laboratory Animals Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal, Resources, National Academy of Sciences - National Research Council.

TABLE OF CONTENTS

I. Production of toxin S *in vitro* and *in vivo*.

A. Introduction

To our knowledge no previous studies on the production of toxin S by *P. aeruginosa* have been done. However, several previous studies have shown that toxin A is not produced constitutively by A^+ toxinogenic strains of *P. aeruginosa* (1-3). Thus it seems likely that S is also not produced constitutively by *P. aeruginosa* strain 388 (Ps 388). However, in order to purify S it is important to consistently produce maximum yields of S *in vitro*. Furthermore in order for S to be considered as a virulence factor, it is necessary that it be shown to be produced *in vivo*.

In the present investigation production of S was followed by quantitating the amount of ADP-ribosyl transferase activity (enzyme activity), in Ps 388 culture supernatants and in sera and skin extracts of Ps 388 infected animals. Production of S *in vitro* was correlated to bacterial growth and in some instances to the production of proteases. A variety of media, media supplements and culture conditions were compared.

B. Materials and Methods

1. Standard culture media. The standard culture medium used was a modification of that previously described by Liu (2). This consisted of the dialysate from Trypticase soy broth, 0.05M monosodium glutamate, 1% glycerol, and 10mM nitrilotriacetic acid (NTA) pH 7.2 (TSBD+NTA). A 25 ml amount of this medium in a 500 ml Erlenmeyer flask was inoculated with an overnight culture of *P. aeruginosa* strain 388, (Ps 388) to an initial cell density of approximately 5×10^7 cells/ml. The culture was incubated at 32° on a reciprocating shaker for 22 hr. The culture supernatant fluid was obtained by centrifugation at 10,000 x g for 20 min at 4°.

2. Deferration and iron determinations. Medium was deferrated to obtain an iron concentration of 0.05 ug/ml by adding 2 ml of 50% CaCl - 2 H_2O per liter, boiling for 5 min in a water bath and filtering through Whatman no. 1 filter paper to remove the precipitate. Residual iron in the medium was determined as described by Mueller and Miller (4). To obtain known concentrations of iron in the deferrated medium, standard sterile solutions of $FeSO_4 \cdot 7H_2O$ were added.

3. Organism. *P. aeruginosa* strain 388 was obtained from Dr. B. Minshew, Seattle, Washington. It was originally isolated from a burn patient at the U. of Washington, Seattle, Washington. This strain produces no detectible extra or intracellular toxin A but does produce large amounts of toxin S which may be recovered from culture supernatants. Ps 388 was stored lypholized and in skim milk at -70°.

4. Growth of Ps 388. Bacterial growth was monitored by aseptically removing a portion of the culture and reading the optical density at 540 nm in a Beckman spectrophotometer 20 (OD_{540}). Initially a portion of each aliquot was diluted in sterile saline and plated on trypticase soy agar plates in order to determine the number of colony forming units (CFU). The CFU was correlated to the OD_{540} and a standard curve obtained for use in future experiments. Protein was determined by a previously described modification of the Lowry method (5).

5. ADP-ribosyl transferase activity. Crude extracts were prepared from wheat germ as described by Chung and Collier (6). ADP-ribosyl transferase activity was measured by the incorporation of radioactivity from [adenine-^{14}C] NAD^+ into trichloroacetic acid precipitable material in the presence of crude wheat germ extracts as previously described (7). Unless otherwise noted, the reaction was performed at 25° in 0.1 ml of 50 mM Tris-HCl (pH 7.0); 1 mM EDTA; 50 mM dithiothreitol; 0.12 mM [adenine-^{14}C] NAD^+ (10.6 mCi/mmole); wheat germ extract containing 150-160 µg proteins and various dilutions of Ps 388 culture supernatant column fractions, mouse serum or mouse skin extracts. The reaction was stopped by the addition of 0.1 ml 10% trichloroacetic acid, the precipitates collected, washed and counted as previously described (7).

6. Animal Experiments. A previously described modification (8) of the burned mouse model developed by Stieritz and Holder (9) was used. Female Swiss Webster mice, NIH/Nm ci CV strain weighing 20±2g were used in all experiments. Viable Ps 388 suspended in 0.5 ml saline at the desired concentration (usually 2 LD_{50}) were injected subcutaneously in the burn site immediately following the 10 sec. alcohol burn. Animals were bled by cardiac puncture then sacrificed at various times after infection. Full thickness specimens of burned skin were removed, weighed and homogenized as described by Sayman et al. (10).

7. Protease determinations. Protease activity was assayed according to Wretland et al. (12), using heat-denatured casein as substrate. The extent of proteolysis was determined by reading the absorption of perchloric acid soluble degradation products at 280 nm. The reaction mixture consisted of casein (10 g/l) and sodium phosphate buffer (0.05 M, pH 7.4) supplemented with $CaCl_2$ (1 mM).

C. Results

1. Effect of ammonium sulfate or nitrolotriacetic acid on Ps 388 growth and yields of toxin S and proteases. Preliminary work in our laboratory showed that the addition of nitrilotriacetic acid (NTA) was mandatory for high yields of toxin S in cultures of P. aeruginosa strain 388. NTA has been shown to inhibit proteases in cultures of P. aeruginosa, however, we do not yet know if this is the exclusive means by which NTA facilitates increased yields of exoenzyme S in cultures of strain 388. Figure 1 shows the effect of varying concentrations of NTA in the culture media on the yields of toxin S by strain 388. Notice that there is a broad range of NTA concentrations at which high yields of toxin S are found. However, at 40 mM NTA yields of S decrease markedly yet growth of Ps 388 is not markedly affected. In order to determine if NTA increased yields of S because it decreased protease activity, we examined the effect of varying the concentration of NTA from 0-4mM on protease activity and yields of S. As shown in Figure 2, protease activity was negligible when the NTA concentration in the growth medium was 1 mM or greater, however, S yields continued to increase (markedly) with increasing concentrations of NTA. Based on the results of these experiments (Figs. 1 and 2) we chose an NTA concentration of 10 mM for future experiments and our standard media will hereafter be referred to as TSBD+NTA.

Ammonium sulfate $(NH_4)_2SO_4$ also has been reported to inhibit Pseudomonas protease activity (2). We added various concentrations of $(NH_4)_2SO_4$ to our

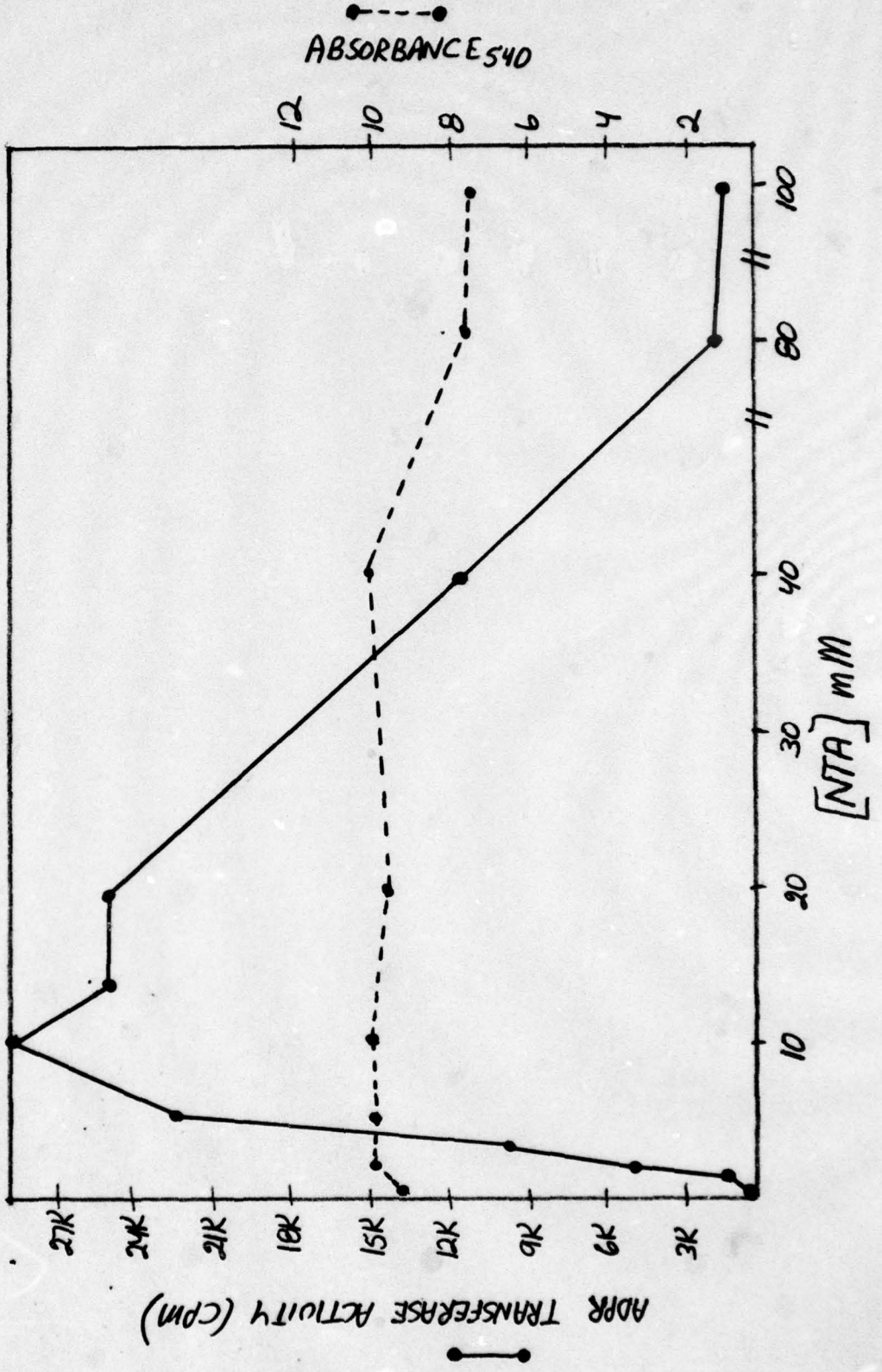


Figure 1. The effect of various concentrations of nitrilotriacetic acid (NTA) on yields of toxin S (●—●) and growth (●- - -●) of Pseudomonas aeruginosa 388.

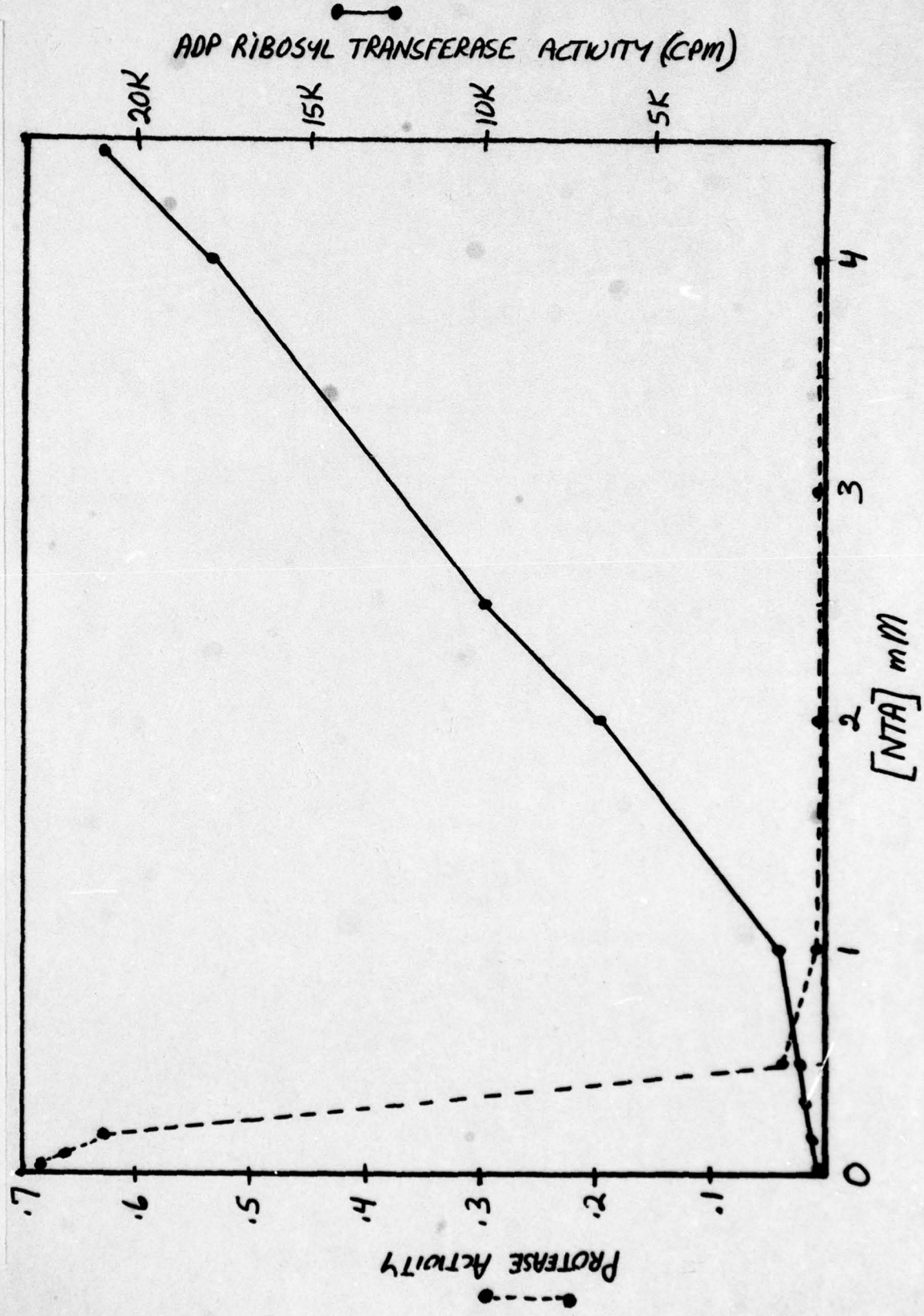


Figure 2. The effect of various concentrations of nitrilotriacetic acid (NTA) on yields of toxin S (●—●) and protease (●- - -●) in cultures of Pseudomonas aeruginosa 388.

standard TSBD media in place of NTA to determine if this would increase yields of S. The results of these experiments (Table 1) indicate that S is not produced (in detectable amounts) in the absence of NTA even when as much as 200 mM $(NH_4)_2SO_4$ is added to inhibit protease activity.

2. Effect of Bovine Serum Albumin and Glycerol on Yields of S. Liu (2) showed that the addition of crystalized bovine serum albumin (BSA) to trypticase soy broth dialysate medium enhances toxin A yields in cultures of strain PA-103. However, the addition of a relatively impure BSA (fraction V) preparation caused a decrease in toxin A yields while markedly stimulating bacterial growth (2). Presumably an inhibitor(s) of toxin A production is found in the crude BSA preparation (2). The effect of adding either pure or crude BSA (fraction V) to TSBD+NTA on toxin S production and bacterial growth was determined. As shown in Figure 3 both crude BSA (fraction V) and crystaline BSA slightly stimulated the growth of Ps 388 but markedly reduced yields of S.

Lui also reported that a glycerol concentration of 1% increased exotoxin A yields by PA 103 (2). We found that the addition of glycerol does not affect toxin S yields by strain 388, although it does stimulate an increase in bacterial growth (Table 2). However, the cell pellets from cultures grown in the absence of glycerol were found to be very mucoid and hydroscopic, thus making them difficult to work with. For this reason, we routinely add glycerol (1%) to our culture media(TSBD+NTA)before inoculation.

3. Effect of various amino acids on yields of S. Lui noted that the addition of monosodium glutamate (MSG) to the culture medium increased the yields of toxin A in cultures of strain PA 103 (2). Thus, we studied the effect of MSG on toxin S yields. The results are shown in Figure 4. Notice the broad range of MSG concentrations at which toxin S is produced in high yields. When alanine or aspartic acid was substituted for MSG the yields of S were markedly reduced. Thus, we now add MSG (100mM) to our growth medium for production of S.

4. Effect of iron on yields of S. We recently reported (3) that the yields of toxin A are inversely proportional to the concentration of iron in the growth medium. Similarly we have found (Fig. 5) that yields of S are inversly proportional to the concentration of iron on the culture medium. Thus the iron concentration of our medium is kept at or below 0.05 µgm iron/ml by deferrating the medium as described above (see section IB).

5. Effect of aeration, temperature and medium pH on yields of S. With regard to the effects of environmental factors on the yields of toxin S we have found that aeration results in increased yields of toxin S when compared to stationary cultures, and that a temperature of approximately 32^{o} is optimal for S production (Table 3). The starting pH of the media is not critical within the range of pH 6.2 to 8.0 (Table 4).

6. Correlation of S yields to the growth cycle of Ps 388. In order to determine when we should harvest Ps 388 cultures to obtain maximum yields of S we correlated yields of S to growth of Ps 388. The results of a typical experiment are shown in Figure 6. Notice that toxin S activity was first detectable at 10 hours and continued to increase until approximately 20 hours, at which time the yields of toxin S appear to level off. It seems therefore, that maximal

TABLE 1

The Effect of Various Concentrations of Ammonium Sulfate on Yields of Toxin S.

% $(NH_4)_2SO_4$	O.D.$_{540}$	CPM/10λ
0 (0mM)	6.0	0
0.1 (8mM)	6.3	0
0.5 (40mM)	5.4	0
1.0 (80mM)	5.1	0
2.5 (200mM)	4.2	0
0+ 10mM NTA	5.4	27,473

Figure 3. The effect of crude (fraction V) or Crytaline Bovine Serum (BSA) on yields of toxin S and growth of Pseudomonas aeruginosa 388. Yields of S with fraction II BSA (▲- - -▲) or with crystaline BSA (△——△); bacterial growth with fraction V BSA (●- - -●) or with crystaline BSA (o——o).

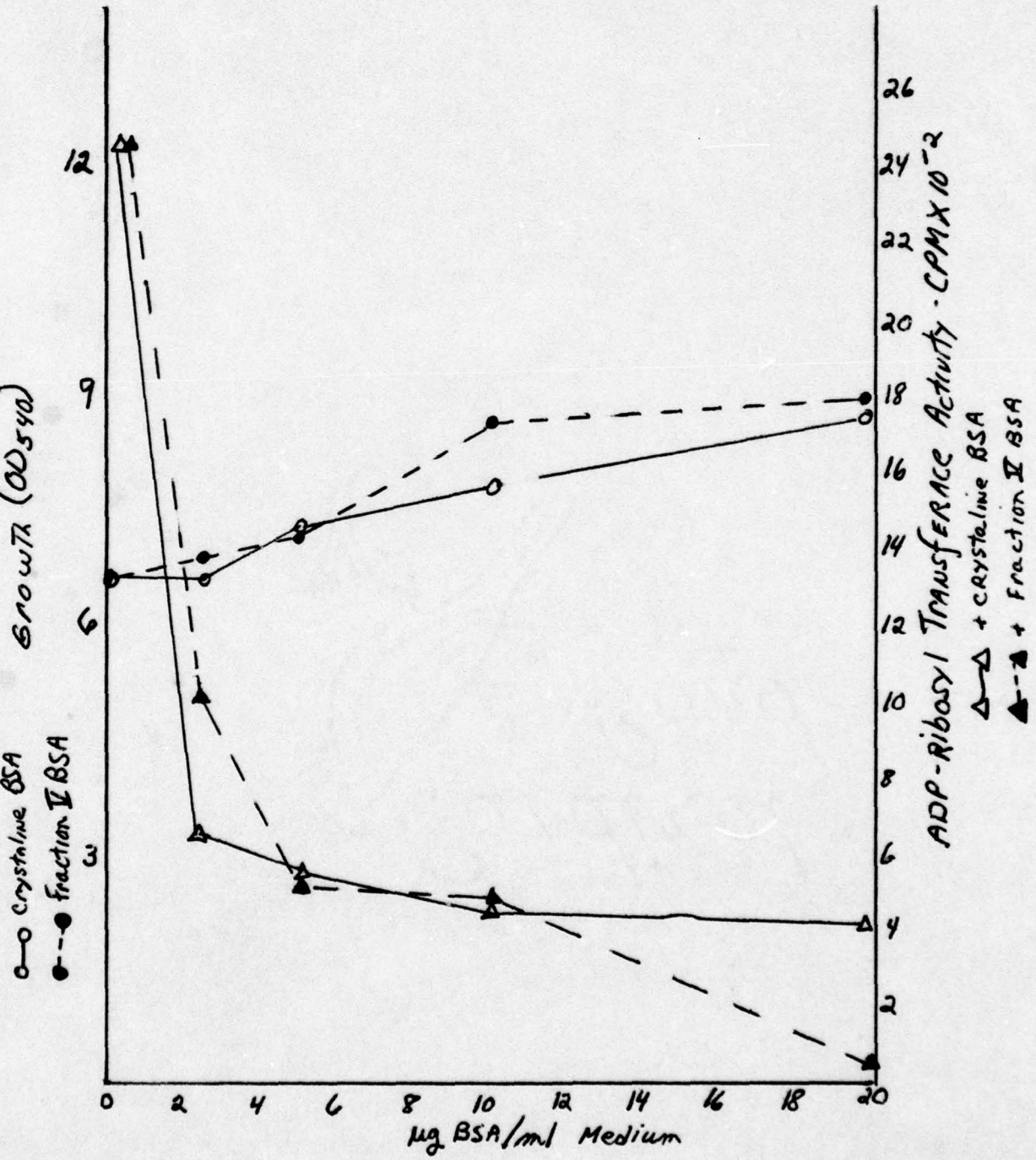


TABLE 2

The Effect of Various Concentrations of Glycerol on Yields of Toxin S

% Glycerol	OD_{540}	CPM/10µl
0	8.4	32,907
0.1	9.6	35,150
0.5	11.1	32,010
1.0	10.2	29,773
3.0	11.7	31,818

Figure 4. The effect of various concentrations of monosodium glutamate (MSG) on yields of toxin S (●—●) on growth (△- -△) of strain Ps 388.

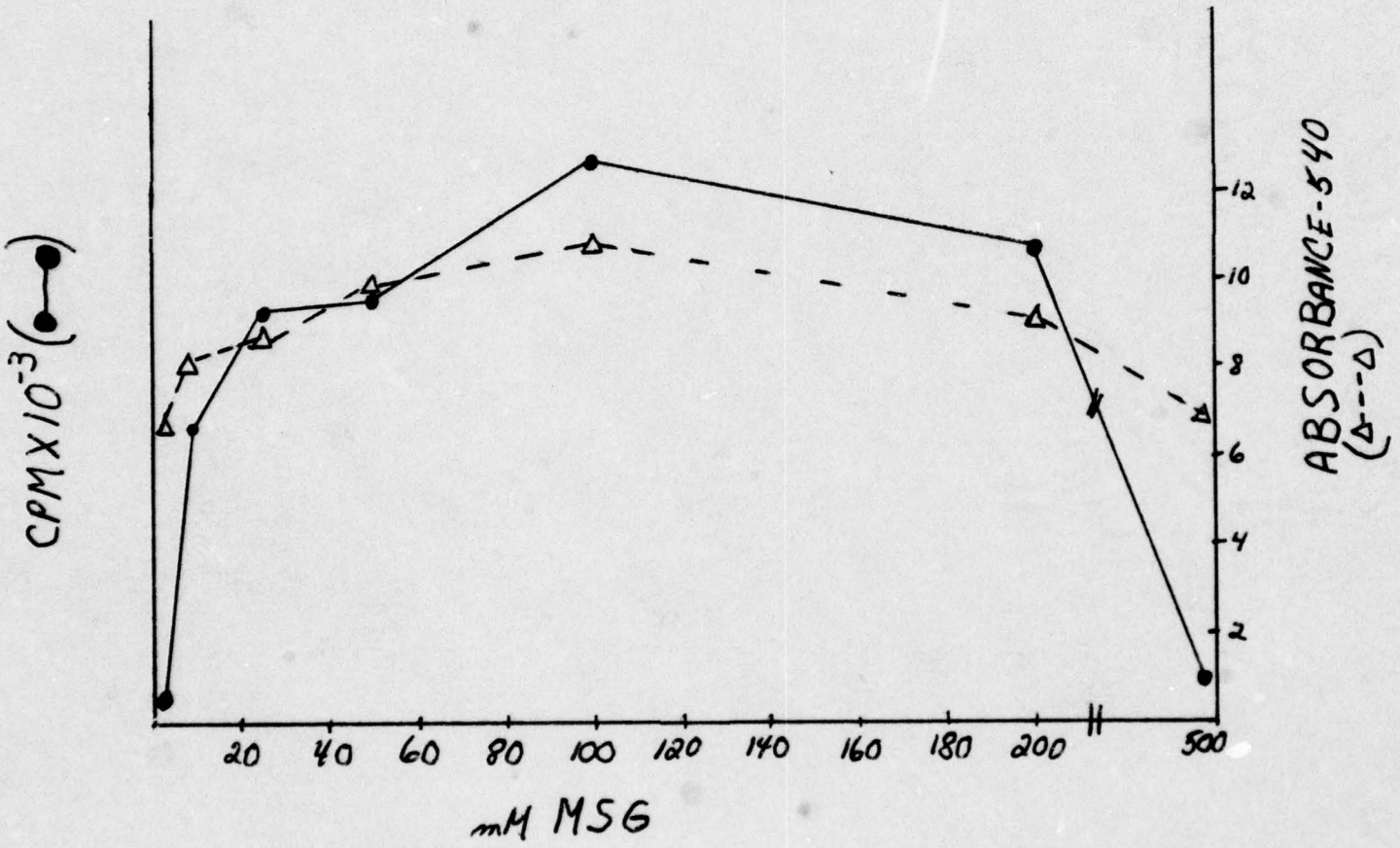


Figure 5. The effect of various concentrations of iron on yields of toxin S (●—●) on growth (▲- -▲) of strain Ps 388.

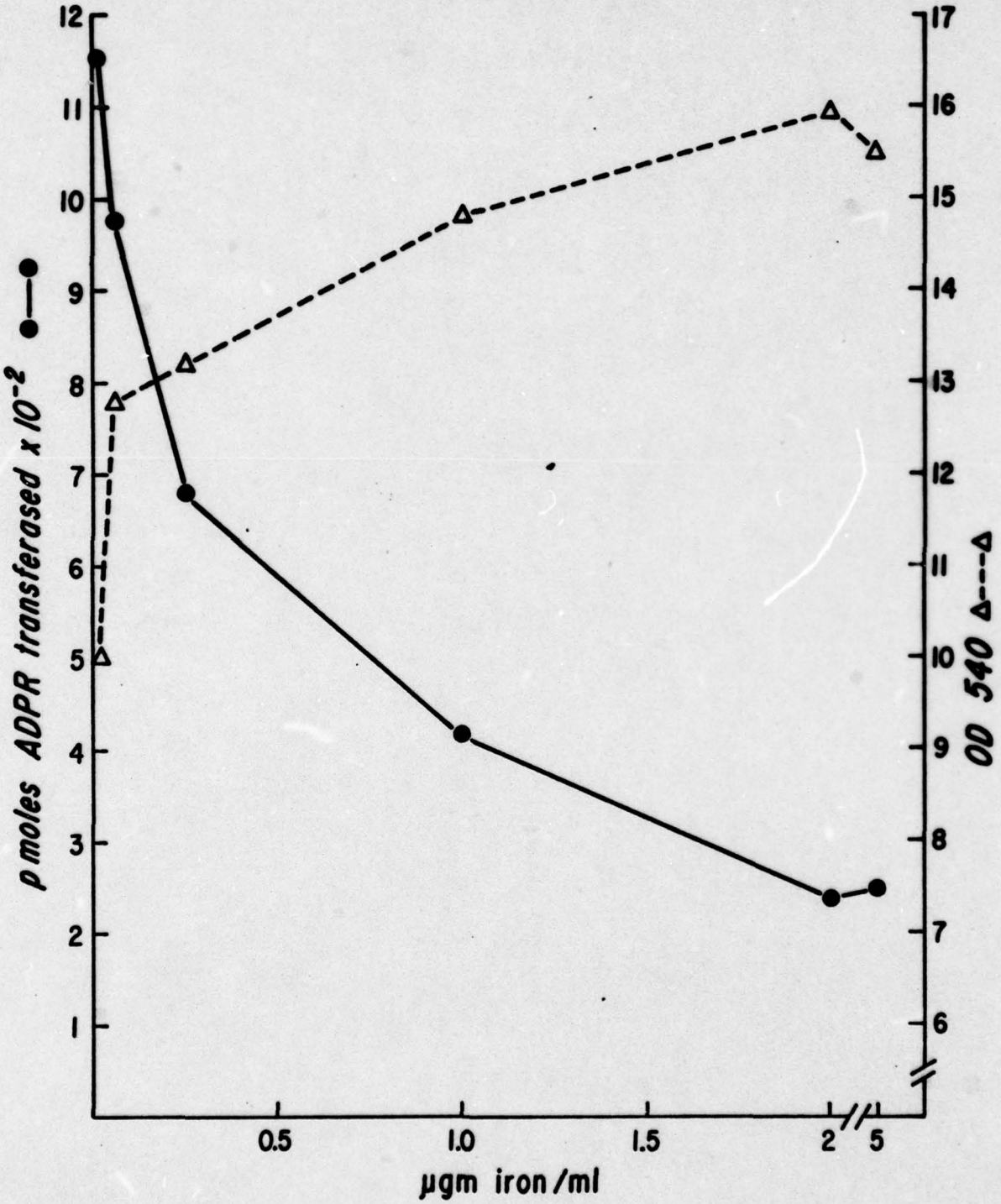


TABLE 3

The Effect of Aeration and Temperature

on S Toxin Yields

Stationary Cultures		
Temp. C	O.D.540	ADPR-Transferase activity (CPM)
25	3.6	724
30	4.2	10,087
32	3.9	3,658
35	5.7	6,798
37	3.9	1,186

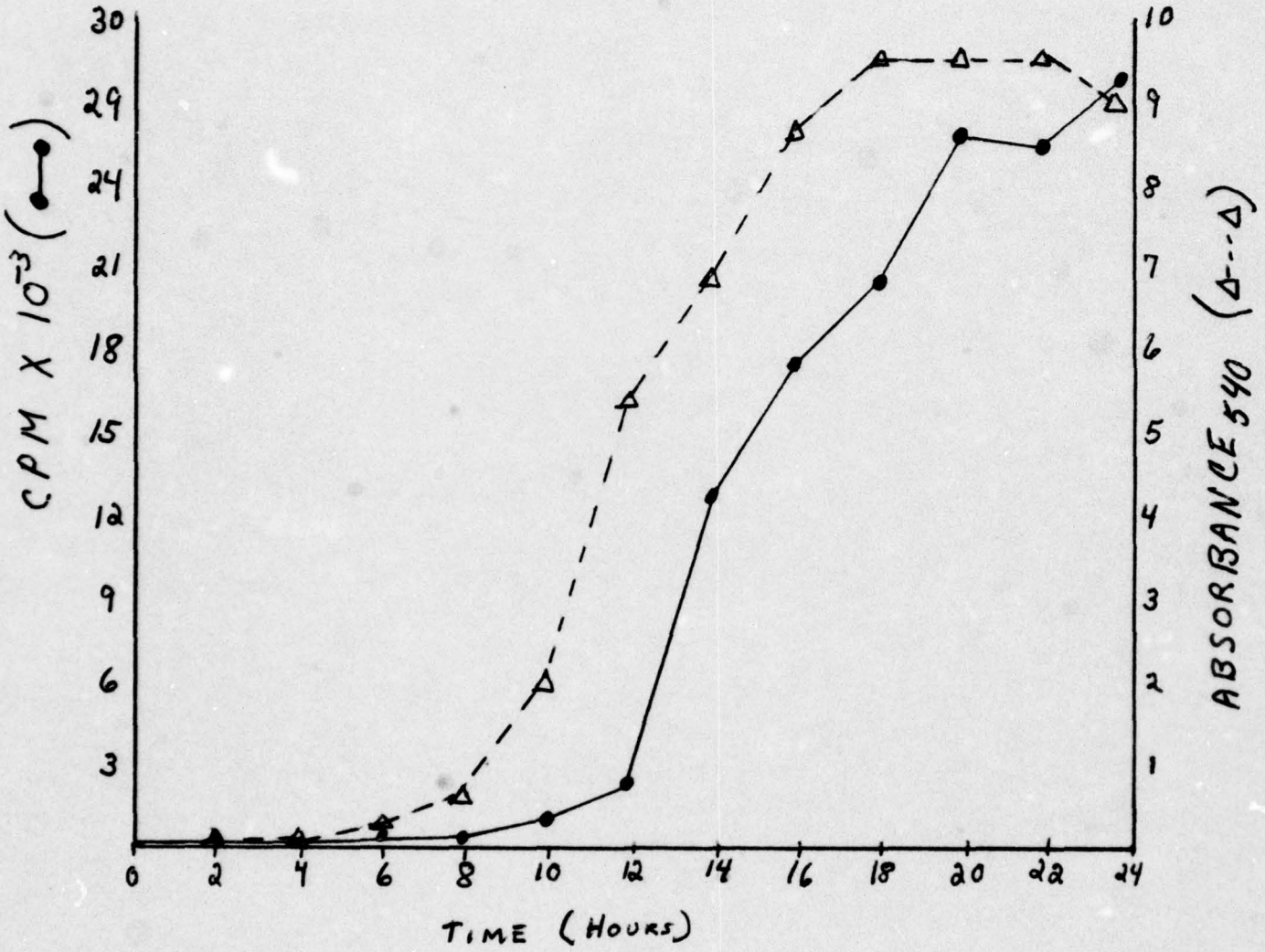
Shaking Cultures		
Temp. C	O.D.540	ADPR-Transferase activity (CPM)
25	9.0	13,980
30	10.8	18,859
32	12.0	19,286
35	10.8	18,768
37	8.1	12,981

TABLE 4

The Effect of Initial pH of Growth Medium (TSBD+NTA) on Yields of S

Starting pH	O.D.$_{540}$	22 hr pH	CPM/10µl
6.25	12.6	8.1	30,049
6.50	12.0	8.0	29,505
6.75	11.4	8.2	32,139
7.00	10.8	8.2	27,382
7.25	10.8	8.1	31,789
7.50	11.1	8.2	36,235
7.75	13.2	8.1	20,100
8.00	8.7	8.2	25,770

Figure 6. S toxin (ADP ribosyl transferase) production during growth of strain Ps 388.



yields of toxin S are found in stationary phase cultures. Currently, we use 20 hour cultures for our experiments on toxin S.

7. Comparison of S yields in various media to that in trypticase soy broth dialysate. The ability of Ps 388 to produce S in culture media that have been used for the production of high yields of other bacterial exotoxins has been explored. Of those media tested, the dialysate from trypticase soy broth appeared to be best for obtaining high yields of S (Table 5). Although other media supported better growth of Ps 388, trypticase soy broth dialysate resulted in the best yields of toxin S. It should be noted that all media (Table 5) were supplemented with 10 mM NTA and 100 mM MSG. When NTA was omitted from any of these media little or no S could be detected.

8. Production of S in vivo. In order for a bacterial product to be considered a potential virulence factor it is necessary to establish that it is produced in vivo during infections. We have established experimental infections of burned mice with Ps 388. As shown in Table 6 the LD_{50} of Ps 388 is markedly reduced by the sublethal burn induced in these animals. When compared to our previous data (8) with strains of P. aeruginosa which produced toxin A but not S, or one (WR-5) which produced neither A nor S, Ps 388 which produces S but not A is quite virulent in this burned mouse model.

In order to determine if S is produced in vivo burned mice were infected subcutaneously with $2LD_{50}$ Ps 388, bled and sacrificed at 18, 24, 36 and 48 hrs post infection. The amount of ADP-ribosyl transferase activity in serum or skin extracts was determined (see methods section IB) and compared to the amount of ADP-ribosyl transferase activity in serum and skin extracts of burned and unburned uninfected animals. The results of these experiments are shown in Figures 7 and 8. The amount of ADP-ribosyl transferase activity in the serum of Ps 388 infected animals is markedly increased (over controls) by 24 hrs. post infection and continues to increase thru 48 hrs. post infection. All infected mice died by 52 hrs. post infection (Fig. 7). The amount of enzyme activity in skin extracts from Ps 388 infected animals was markedly elevated by 18 hrs. post infection, increased slightly at 24 hrs. then remained constant thru 48 hrs. post infection (Fig. 8). Since a small amount of endogenous ADP-ribosyl transferase activity was found in the serum of uninfected control animals (Fig. 7) the specificity of the enzymatic activity was determined by preincubating samples with normal rabbit serum, rabbit anti S or rabbit anti A antisera. As is shown in Table 7 the enzymatic activity in the serum and skin extracts from Ps 388 infected animals was neutralized by preincubation with anti S serum but not normal rabbit or anti A sera. Thus this enzymatic activity is specifically due to the presence of toxin S. On the other hand the low enzymatic activity found in serum of non-infected animals is not neutralized by any of the sera tested and is therefore due to endogenous mammalian enzymes.

D. Discussion

In summary, these data indicate that toxin S is not produced constitutively by strain Ps 388; that all conditions which maximize growth do not correlate with maximum toxin S yields and that the optimum conditions for production of toxin S are not the same as those for toxin A production. Furthermore, Ps 388 is highly virulent in a burned mouse model and specific toxin S can be detected in the serum and skin extracts from these infected animals. Thus toxin S is produced in vivo during experimental P. aeruginosa infections.

TABLE 5

Toxin S Yields in Media of Varying Composition

Medium[a]	O.D.$_{540}$[b]	ADP-ribosyl transferase Activity (C.P.M./10µl)
Tripticase soy broth dialysate	5.1	35,835
Tripticase soy broth	10.9	18,823
Protease peptone dialysate	3.6	6,207
Protease peptone	6.5	13,625
Syncase	10.1	10,089
PGT	11.7	267

[a] All media were supplemented with 10mM NTA and 100 mM MSG.

[b] Bacterial growth as measured by absorbance $_{540nm}$ of 20 hour cultures.

TABLE 6

A Comparison of the LD_{50} of Ps 388 (A^-S^+) to Other Strains of *P. aeruginosa* in Normal and Burned Mice[1]

Strain	Toxin A	Toxin S	LD_{50} (CFU)[2] Normal Mice	LD_{50} (CFU)[2] Burned Mice
Ps 388	-	+	2.0×10^6	1.1×10^2
PA 103[3]	+	-	1.8×10^6	1.2×10^3
PA 86[3]	+	-	2.5×10^6	2.3×10^3
WR 5[3]	-	-	2.4×10^7	7.5×10^6

[1] Groups of 10 mice were injected with each dilution of each *P. aeruginosa* strain tested.

[2] CFU, Colony Forming Units.

[3] See reference 8.

Figure 7. A comparison of the ADP ribosyl transferase activity in serum from burned mice infected with strain Ps 388 to that in serum from uninfected burned or non burned uninfected mice.

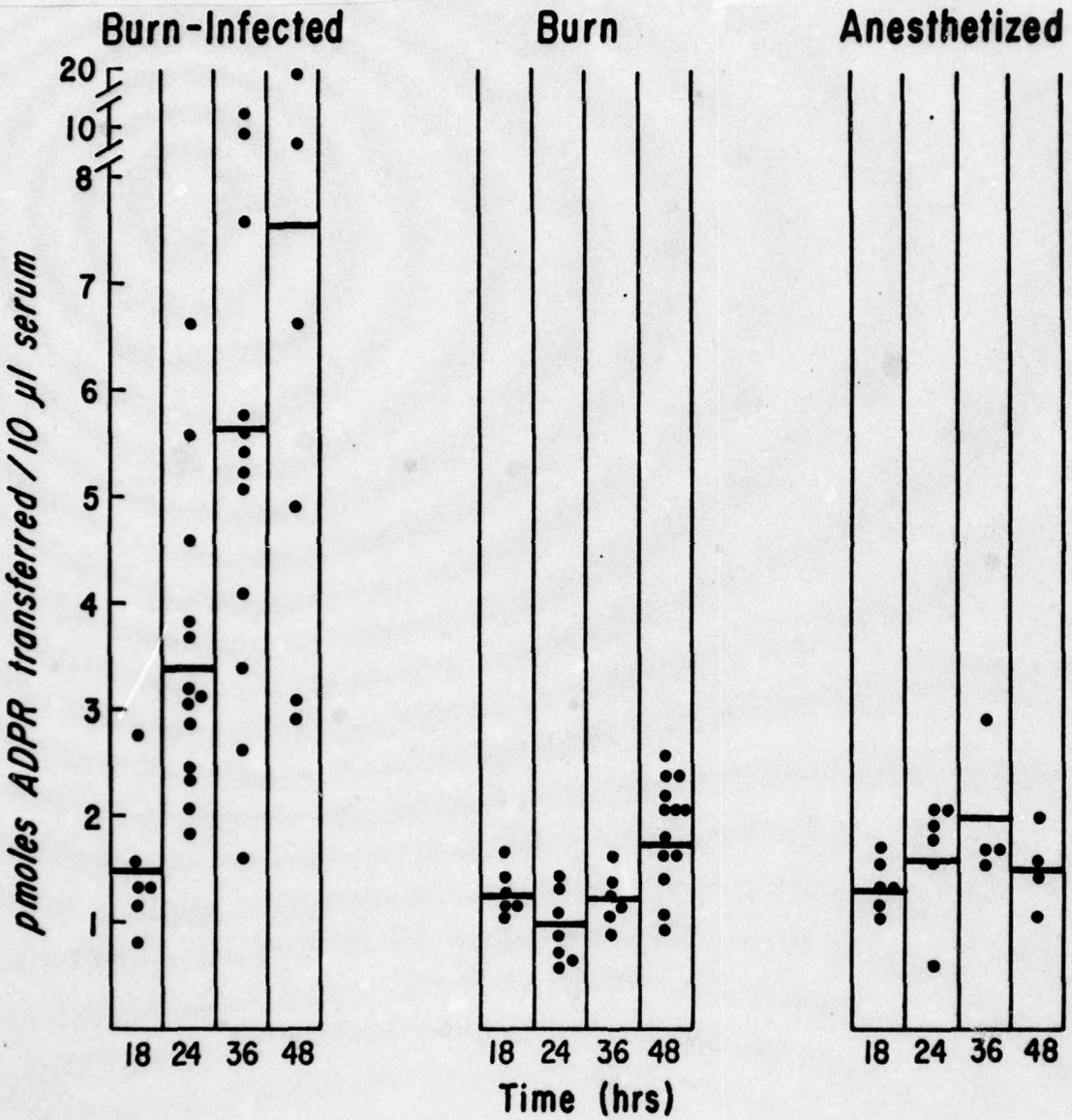


Figure 8. A comparison of the ADP-ribosyl transferase activity in skin extracts from burned mice infected with strain Ps 388 to that in skin extracts from uninfected burned or non burned uninfected mice.

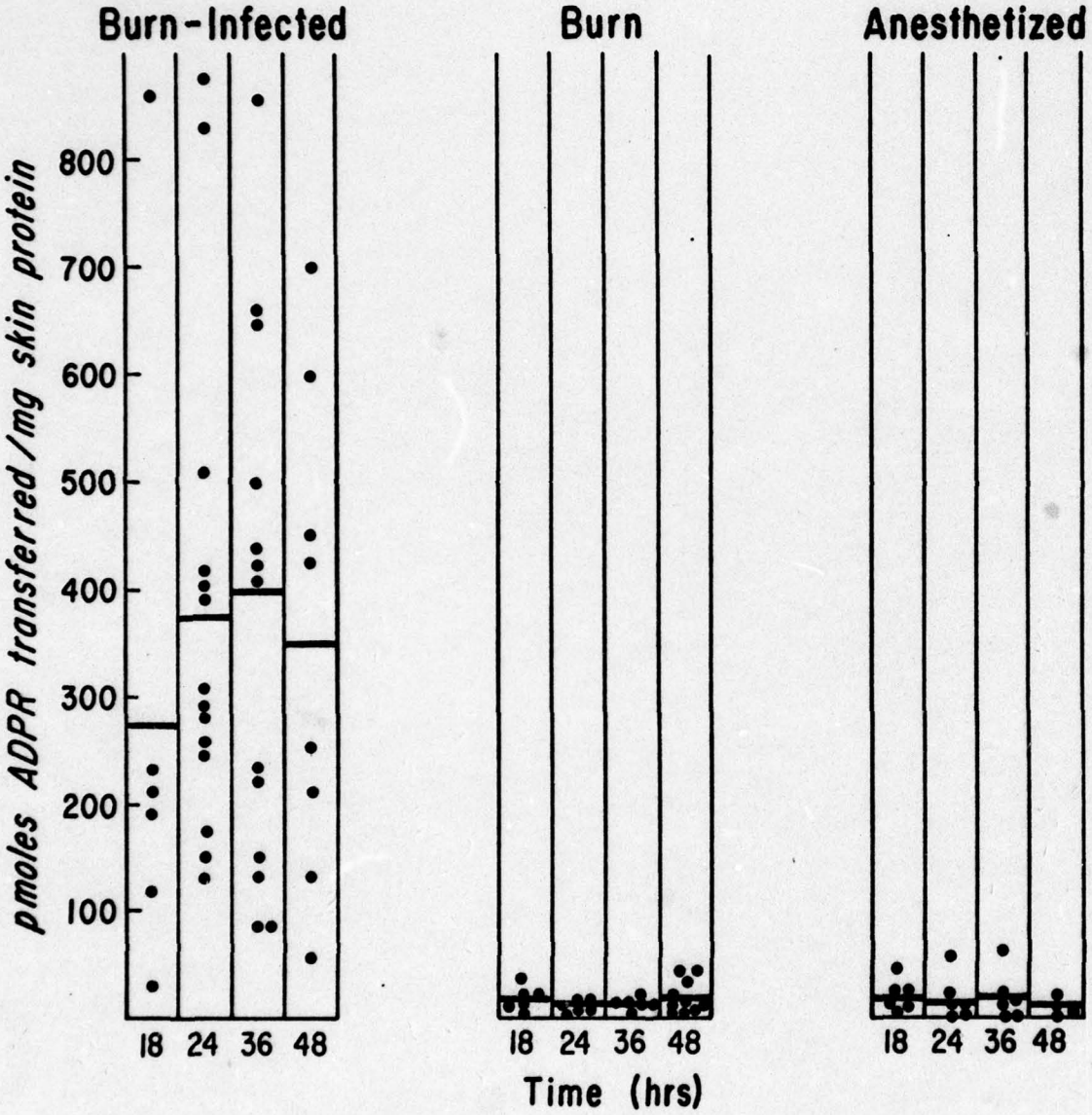


TABLE 7

Neutralization of ADP-ribosyl Transferase Activity from Serum and Skin Extracts of Burned Ps 388 Infected and Uninfected Mice[1]

Sample	Counts Per Min.		
	NRS[2]	Anti A[2]	Anti S[2]
Burn Infect. Serum (24 hr PI)	744	841	98
Burn Infect. (36 hr PI)	1,400	1,306	178
Burn Uninfect. Serum (24 hr.)	200	252	230
Burn Uninfect. Serum (36 hr.)	271	244	256
Anesthetized Normal Serum (24 hr.)	231	254	251
Anesthetized Normal Serum (36 hr.)	882	998	863
18 hr. Burn Infected Skin	2,362	2,277	287
36 hr. Burn Infected Skin	3,298	2,556	582
48 hr. Burn Infected Skin	1,922	1,820	241
36 hr. Burn Infected Skin	48	52	50

[1] Samples in 10µl were preincubated with 10µl serum for 15 min at 37°C then immediately assayed for ADP-ribosyl transferase activity.

[2] NRS, normal rabbit serum; anti A, rabbit toxin A antiserum; anti S, rabbit S antisera.

II. Purification and Characterization

A. Introduction

The initial investigation into a purification scheme for toxin S has demonstrated that it is possible to enrich toxin preparation from culture supernatants by chromatographic and electrophoretic techniques. Our studies have concentrated on the following:

1. To isolate a protease deficient mutant of Ps 388 to facilitate purification;

2. To obtain toxin with a medium scale batch wise procedure from our ion exchanger (DE-52 cellulose) to reduce toxin preparations to a manageable size starting with 16 1. fermenter runs;

3. To determine the feasibility of anti A sepharose affinity chromatography for use as a simple purification step, based on prior observation of interaction with crude culture supernatant preparations of Ps 388.

Characterization of the enzyme has been initiated by observing the behavior of toxin S preparations with typical purification schemes such as gel filtration, (for molecular weight determination) and acrylamide gel electrophoresis; ion exchange (net charge), isoelectric focusing (isoelectric point determination) and by characterization of S by comparison to toxin A.

B. Methods

1. Production of toxin S in large batches. The culture medium used was that previously described above (IB). A flask-to-volume ratio of 20:1 was maintained and starter cultures were inoculated with a 15 hr culture of Ps-388. Overnight and starter cultures were incubated at 32° on a reciprocating shaker (150 linear excursions/min) (Lab-line instruments, Melrose, ILL.). Optical densities were obtained by aseptically removing a portion of the culture and reading the optical density at 540 nM in a Beckman spectrophotometer. Media prepared for 16 1. fermenter runs were autoclaved and cooled in the fermenter immediately before the run was to start. Sixteen liters were inoculated with 700 ml of a starter culture with an OD_{540} = 0.4. A 20 1. capacity microferm fermenter (New Brunswick Scientific, New Brunswick, N.J.) was used for 3 separate 16 1. batches of Ps 388-6. Fermentation conditions were as described by Leppla (15). Cultures were harvested at 16-22 hours. Bacteria were removed by centrifugation with a GSA rotor in an RC-2B Sorvall centrifuge.

2. Isolation of a protease deficient mutant of Ps 388. Protease production by individual colonies was assayed using skim milk plates as described by Wretland et al. (12). Mid log phase cultures of Ps 388 were exposed to either ethyl methanesulfonate (EMS) 0.5% for 60 min. or nitrosoguanidine (NTG) 100 µgm/ml for 60 mins. then centrifuged, washed, resuspended in TSB-D and grown at 32° overnight. Survivors of mutagenesis were plated (approximately 40 colonies/plate) on agar containing 1.5% skim milk. The parent Ps 388 produces sufficient protease 1 and 2 (elastase) to give a clear zone around isolated colonies in 36 hr. whereas strains of P. aeruginosa such as PA 103 which produce

very little protease 1 and no elastase do not cause visible clearing even after 96 hr incubation. Although 35,000 Ps 388 survivors of EMS treatment were examined, no protease - colonies were detected. However, when 30,000 colonies were screened following NTG treatment, 9 potential mutants were identified. Of these, two (Ps 388-5 and Ps 388-6) were shown to be stable and were further examined to quantitate the amount of protease and toxin S they produced. Culture supernatants of Ps 388-6 assayed as previously described (Section IB above) contained 1/40 the protease activity of the parent whereas Ps 388-5 contained 1/10 the protease activity of the parent. Supernatants of Ps 388-6 contained the same amount of toxin S as the parent whereas toxin S activity in Ps 388-5 supernatants was reduced approximately 40%. When retested after being subcultured 10 times Ps 388-6 has remained stable. The toxin S produced by Ps 388-6 appears identical to that produced by Ps 388 in terms of neutralization with rabbit S antisera, enzymatic activity and heat stability. Of particular interest is our observation that Ps 388-6 only produced detectable S when grown in medium containing NTA. Thus the NTA requirement appears to be related to some other effect of NTA besides its inactivation of protease activity.

3. Preparation of antisera and enzyme neutralization. Specific toxin A antiserum was produced in rabbits as previously described (13). Antiserum was also prepared in rabbits against partially purified toxin S. Two rabbits were each injected with 100 µgm partially purified S in complete Freunds adjuvant. The inoculum contained in 1 ml was divided and 0.4 ml given subcutaneously in the rabbits back, 0.4 ml given intraperitoneally and 0.1 ml injected into each of the 2 rear foot pads. Three weeks and 5 weeks after the primary immunization the rabbits were boosted with 100 µgm S in incomplete Freunds adjuvant. Again the inoculum was divided among the 3 injection sites. The rabbits were bled 10 days after the last booster injection. Each of the rabbits were prebled prior to the 1st injection and this prebled sera was used as the normal rabbit sera in these experiments. Unless otherwise indicated, enzyme-inactivation by antibody was determined by assaying the ADP-ribosyl transferase activity (see Section IB) after incubation of toxin A or toxin S with the specified antiserum or normal rabbit serum for 5 min at 37°. The antiserum was diluted in saline containing 0.1 mg/ml BSA.

4. ADPR transferase activity. Substrate-containing enzyme preparations were obtained from wheat germ extracts (6). The ADPR-transferase activity of culture supernatant and partially purified toxin S was measured as described previously (IB).

5. Toxin purification. All purification steps were performed at 5°. Toxin S was removed from culture supernatants by dilution of centrifuged culture supernatants with 3 volumes of distilled, deionized water and subsequent addition of diethylamino-ethyl (DEAE) cellulose (Whatman DE-52, Reeve Angel Biochemicals, Clifton, N.J.). The suspension was stirred 2 hours and toxin depletion from culture supernatant was monitored by enzyme assay. The DE-52 was allowed to settle and the culture supernatant was discarded. The DE-52 was washed with 0.05M NaCl, 0.01M Tris pH 8.0 and toxin was obtained in various salt elutions in a Buchler funnel. Enzymatically active material was precipitated with 70% ammonium sulfate and stored either a) frozen (-70°) after resuspension in 0.01M Tris pH 8.0 with no dialysis or b) frozen (-70°) after resuspension in 0.01M Tris, pH 8.0 with 10mM DTT and dialysis against 2 changes of 5 mM DTT containing

buffer. These stock preparations of various salt elutions from the DE-52 cellulose were used in all subsequent purification procedures.

6. Gel filtration. Gel filtration of toxin S was preformed on Sephadex G-100 and Biogel A .5M columns previously equilibrated with a variety of buffered solution allowing elution in the presence of detergent, reducing agents or high salt. Columns were run at 4° or 24°.

7. Hydroxylapatite chromatography. Hydroxylapatite (Biogel HT, Bio Rad, Richmond, California) columns were packed in 5 mM Potassium Phosphate, pH 7.0 and linear gradients to 400 mM phosphate were used for separation of proteins. Columns were run at 4°.

8. DEAE-cellulose/DEAE sephadex chromatography. Ion exchange columns packed with DEAE-cellulose (DE-52) or DEAE sephadex (Pharmacia A-25) were equilibrated with a variety of buffers including or excluding protease inhibitors. Linear gradients in NaCl were used after binding toxin to previously washed ion exchanger. Enzymatically active regions were pooled and concentrated with PM-10 Amicon membranes. These concentrated solutions were frozen at -70° for further purification

9. Isoelectric focusing. A LKB 110 isoelectric focus column was used to purify toxin S from partially purified material (post ion exchange). pH 3-10, 3-5 and 4-6 ampholytes have been used to generate maximum resolution of toxin from other proteins. 2M urea was incorporated in preparative scale isoelectric focus experiments. Active fractions were pooled and frozen at -70°. OD_{254} and pH were automatically measured throughout elution from the column.

10. Analytical polyacrylamide gel electrophoresis and isoelectric focus gel electrophoresis. The method of Laemmli was used for SDS-polyacrylamide gels in both slab and cylindrical gel electrophoresis(17). 12% and 15% gels were routinely used to follow purification of toxin S. Isoelectric focusing was performed with Biolytes (Bio-Rad, Richmond, CA) in 10% gels cast with and without urea. Enzymatic activity was recovered from all cylindrical gels by slicing and eluting the gel slices in .2 ml of 10 mM Tris, pH 8.0±10 mM DTT, or into .2 ml water. The pH of isoelectric focus gel slices was determined by eluting the slices of a parallel gel into water. The pH was recovered after gel slices incubated at 4° overnight. The eluates were maintained at 2°-4° during pH measurement. Parallel gels were stained or sliced.

11. Affinity chromatography. Antibody to exotoxin A was partially purified and coupled to sepharose 4B by the method of Iglewski and Sadoff (7). Toxin S was applied to anti A columns in .01M Tris pH 8.0. Material binding to the column was eluted with 3M KI and immediately desalted on a small G-25 sephadex column. Active material was lyophilized or concentrated on PM 10 membranes and stored at -70°.

C. Results

1. Purification. In an effort to determine a straight forward purification scheme, several procedures were decided upon as constant. a) Enzyme activity

was relied upon as the criterion for the determination of presence of toxin. Assay conditions were adjusted so that an excess of substrate and NAD were available when determining specific activities. b) Removal of toxin S from culture supernatant was preformed by addition of DE-52 to diluted culture supernatant. It was found that the majority of toxin S enzyme activity was bound to DE-52, just as with toxin A; however, a much greater amount of DE-52 is required (at least 100 g/l). Elution of toxin is simple and rapid from the DE-52 cellulose in a Buchler funnel, and yields of toxin are good (Table 8). At the present time the enzyme activity is recovered from the high salt washes of the DE-52 by ammonium sulfate precipitation. We have observed irreversible denaturation with this ammonium sulfate precipitation step and therefore wish to replace this step with ultra filtration as soon as is practical. All further attempts at purification were initiated with material recovered from the post DEAE-post ammonium sulfate pellet. This partial purification scheme is shown in Fig. 9. The approach followed was to run small scale gel filtration, then ion exchange , and lastly preparative isoelectric focusing in an attempt to develop a second purification stage. Initial experiments demonstrated that the enzymatic activity in post DEAE-cellulose material elutes in the void volume of both G-100 sephadex and biogel A.5m. Thus, gel filtration has not yet proven itself either reliable for purification of S or for characterization since enzymatic activity seems to associate promiscuously with other protein (or perhaps LPS) and tends to elute in the void volume of these columns with 100,000 and 500,000 mw exclusion limits, respectively. It is evident that material applied to gel filtration columns will have to be further purified thru stage II before an accurate estimate of molecular weight is feasible. Hence, gel filtration does not at this time appear as a good alternative in the purification scheme for stage III. Analytical gel electrophoresis of fractions from gel filtration demonstrated a large amount of aggregation with virtually all biological activity unable to penetrate a 7 1/2% nondenaturing (i.e. large pore) gel. SDS gels on the other hand demonstrate 2 peaks of activity with this material (Fig. 10). (SDS was found to denature the substrate in the ADP-ribosylation assay at levels above .01%, hence the slice eluates were diluted before assay.) Since separation under nondenaturing conditions was poor, it was decided to procede to the next alternative, a second DE-52 elution, but with a linear gradient of salt and conventional column configuration. This afforded improved separation from contaminating protein and nucleic acid and was simple and economical to run. Similar columns were run with DEAE sephadex with somewhat higher resolution, but both ion exchanges appear reasonable for the second stage of purification. The third alternative we approached for a possible second stage was preparative sucrose isoelectric focusing. Resolution of toxin on preparative sucrose isoelectric focus columns was demonstrated, but a rather large aggregate was formed at about pH 4.0 which may have interfered with the elution of toxin from the column and most certainly decreased the possible column load with material from this stage of purification (i.e. Stage I). Based on these observations it has been decided to keep, as the first 2 stages in purification, a batch elution with DE-52 and a gradient elution from DE-52. Isoelectric focusing shows great promise as a third stage, and we have investigated this as well as gel filtration and hydroxylapatite chromatography.

It was observed that material frozen at -70^{o} that was partially purified by DE-52 bath elution was stablilized by dialysis vs. a buffer containing DTT.

TABLE 8

Toxin S Activity: 16 1 Fermenter Run #2

	Material	Protein gm	Toxin Activity Recovered %	Purification Fold
1.	Culture supernatant	4.8	100	1
2.	DE-52 Bound (total)	1.6	80	2.4
3.	Stage I, Post .15m NaCl Wash	.48	60	6.0
4.	" .25m " "	.88	20	1.9
5.	" .35m " "	.18	0	-

Fig. 9

Partial Purification Scheme for Toxin S from 16 l Fermentation of 388-6.

The final product is termed stage I toxin ± DTT.

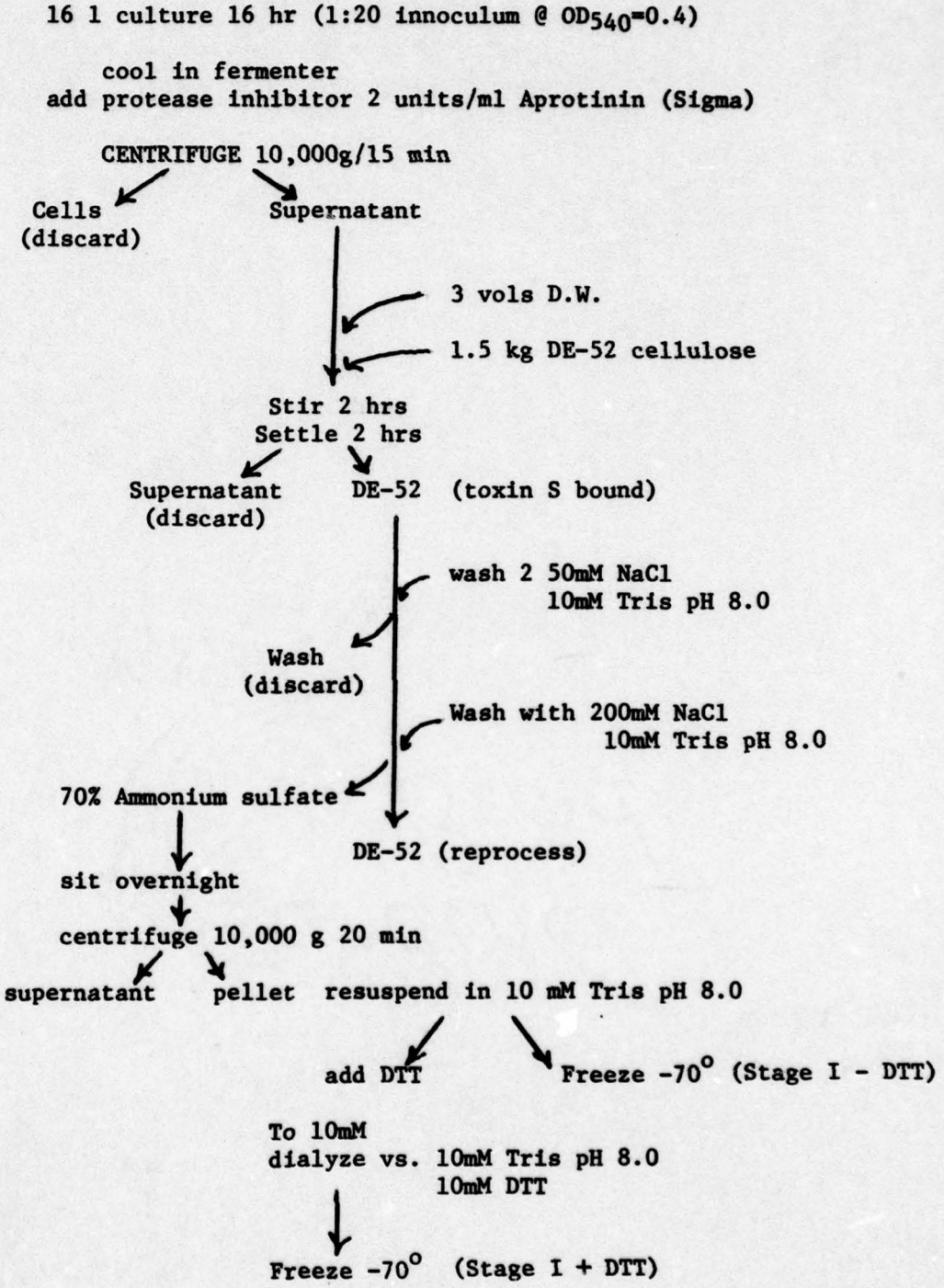
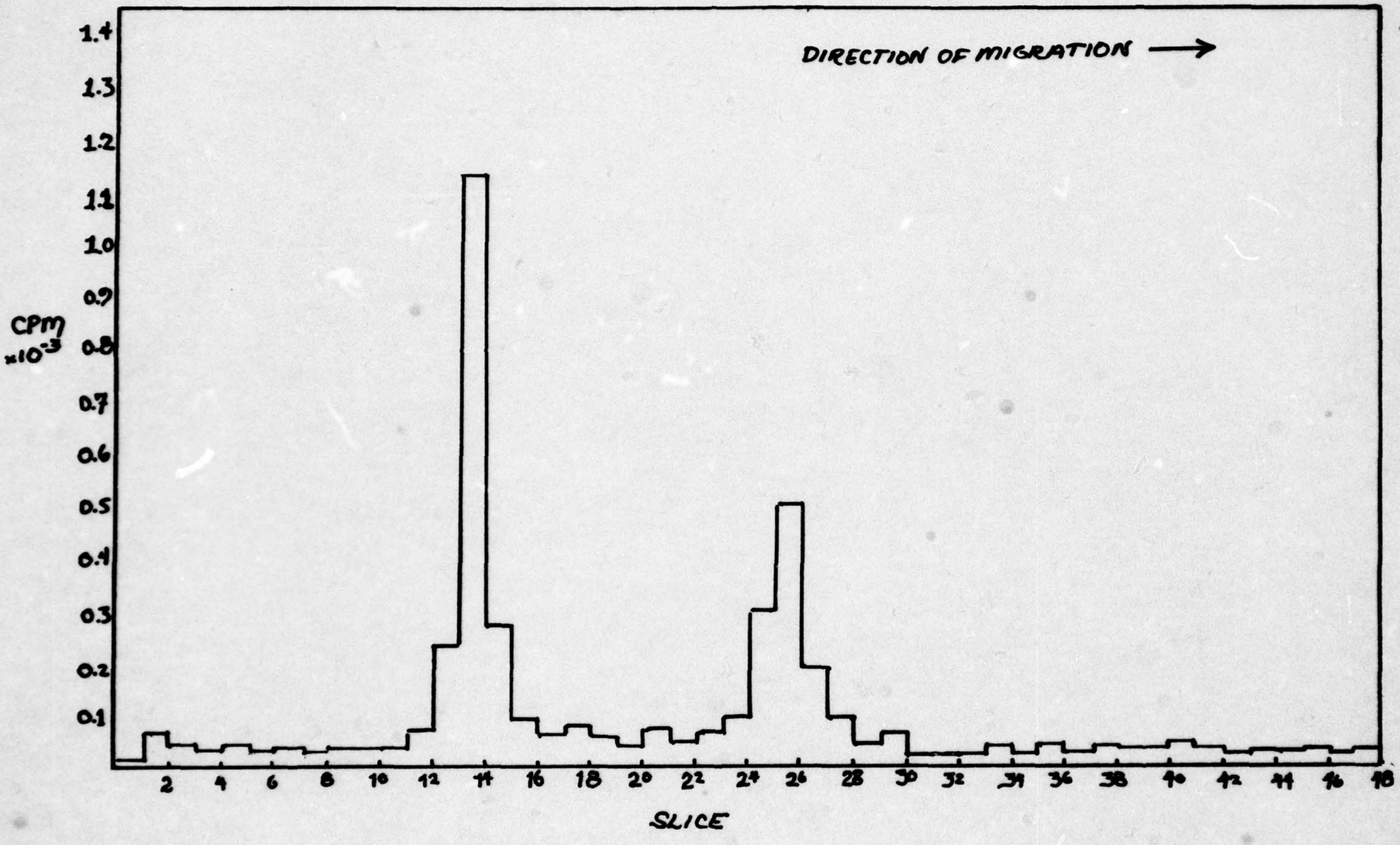


Figure 10. SDS Gel Electrophoresis of Toxin S.

50 µg Stage I toxin + DTT was heated at 37° for 30' with SDS prior to electrophoresis. 1.5λ of eluate was assayed from each slice. Parallel gels were stained or contained protein standards. Slices were 1.28 mm.



It was also observed that dialysis of toxin preparations under a variety of other conditions resulted in loss of biological activity (Table 9). Dialysis against any of these buffers in the presence of DTT stabilized the enzymatic activity. Also, pretreatment of S toxin with SDS at low concentration demonstrates an "activation" phenomenon as shown in Fig. 11. These data suggest the possibility that we are able to activate toxin, and that we then have a choice of attempting to purify activated, or non-activated (i.e. proenzyme) toxin. Since there is no way of knowing to what extent our purification scheme might "activate" the toxin it was decided to concentrate on the stablized form, i.e. the enzymatically active DTT or DTT-plus denaturing agent treated toxin. Hence the fermenter batches were split into DTT treated (i.e. dialyzed post ammonium sulfate pellets, resuspended in .01M Tris 10 mM DTT) and non DTT treated fractions. Both preparations have been separated on DE-52 gradients ± DTT, and on gel filtration and gel electrophoresis under similar conditions.

Affinity chromatography was not found a viable step in the purification procedure at this time, since with available antisera, we were unable to bind more than $2x10^3$ pM/min ADP ribosyl transferase activity per ml column volume. This amount of S activity bound irrespective of column loads. It was not possible in these experiments to concentrate the toxin S which bound to the toxin A antibody column and obtain a measurable amount of protein. Thus with available antisera, the small amount of S that is bound is not adequate to allow consideration of this technique for purification at this time.

2. Characterization of S. Pseudomonas toxin A is produced as a toxic proenzyme that is virtually devoid of ADP-ribosyl transferase activity (14,15). The transferase activity of toxin A is expressed when the molecule is cleaved by proteolysis to yield an enzymatically active fragment (12) or when it is denatured and reduced (14,15). In contrast, we found (6,14) that treatment of exoenzyme S with 4 M urea markedly reduced its enzymatic activity. The presence of dithiothreitol slightly modified the effect of urea on exoenzyme S activity (Table 10).

Crude toxin S was initially found to lose activity upon dialysis, although enzymatic activity of culture supernatants was not disturbed by ultrafiltration on a 10,000 MW cutoff amicon membrane. Dialysis against several agents have demonstrated stabilization. However, (Table 9) the presence of DTT at 5-10 mM has been shown to stabilize enzymatic activity, either by inhibition of proteases or by maintaining the toxin in an enzymatically active state. Pretreatment with urea or urea-DTT does not potentiate activity as is the case with toxin A, but actually can have a detrimental effect (Table 10). Unlike urea pretreatment, SDS pretreatment can have a stimulatory effect on ADP ribosylation activity of toxin S (Fig. 11). However, it is necessary to reduce the SDS concentration below .05% in the reaction system previously described in order to prevent substrate denaturation. It is not known at this time what concentration of SDS might be optimal, or what other conditions need to be optimized, but treatment with up to 0.5% SDS has been shown to activate toxin S, if the toxin is diluted before assay to lower the SDS concentration (Fig. 11).

TABLE 9

Stability of Toxin S Enzymatic Activity to Dialysis for 20 hr, 4°

Dialysis Media	% Activity
No Dialysis	100
20mM Tris pH 8.0	1
" + 5mM NTA	5
" + 2mM EDTA	2
" + 1mM DTT	64
" + 2mM DTT	86
" + 5mM DTT	97
" + 10mM DTT	100

Figure 11. SDS pretreatment of 388-6 culture supernatant (toxin S). Toxin S was incubated at 25° for 15 min with an equal volume of SDS in water, then diluted 1:5 and assayed as described in materials and methods (IB).

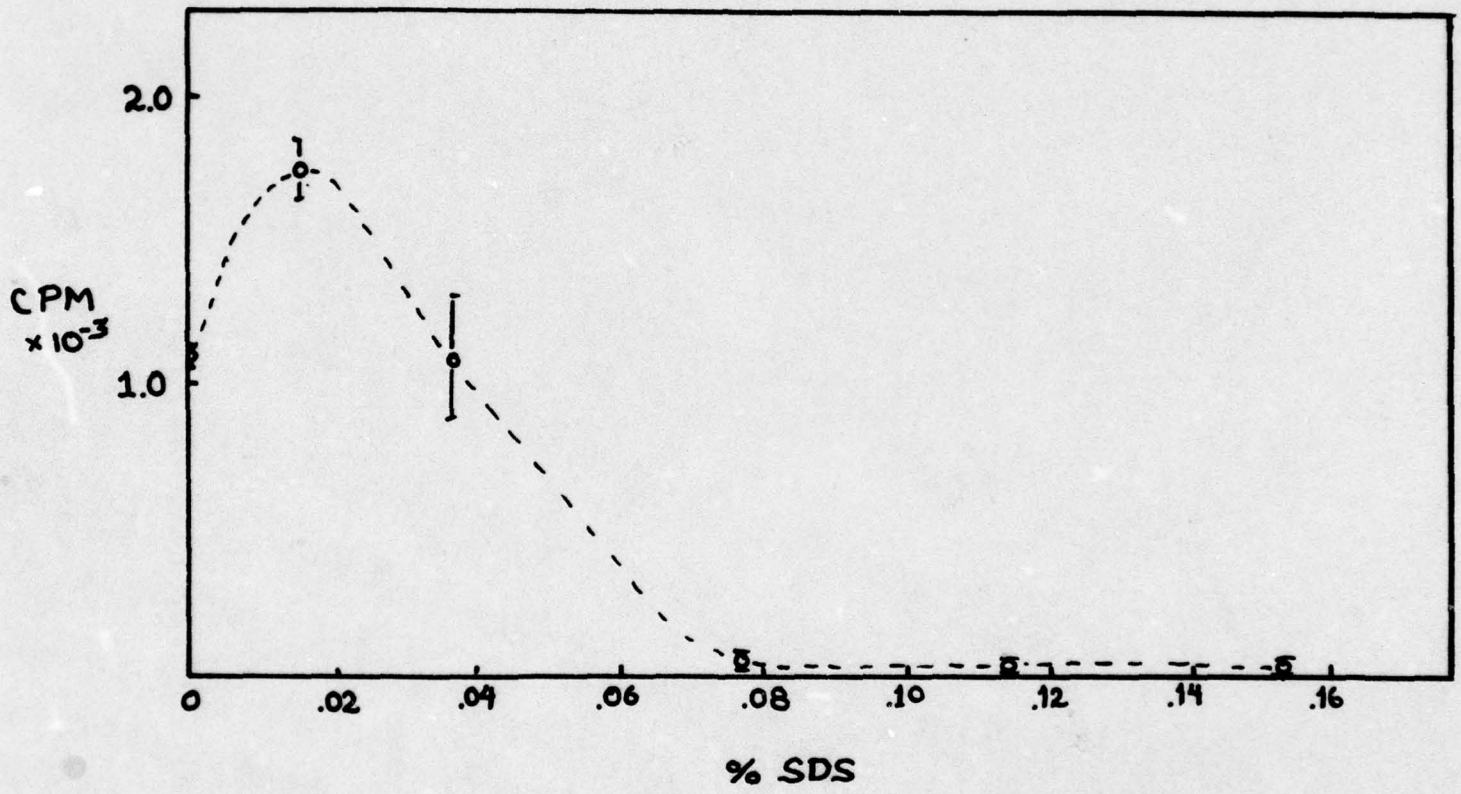


TABLE 10

Effect of Urea and Dithiothreitol on ADP-ribosyl Transferase Activity of Exoenzyme S and *Pseudomonas* Toxin A

Treatment	ADP-Rib Incorporated pmoles	
	Toxin A	Exoenzyme S
H_2O	0.72	1200
1% Dithiothreitol	0.67	1250
4M Urea	10.9	760
4M Urea + Dithiothreitol	26.1	960

Pseudomonas toxin A (.02 µg) or exoenzyme S (5 µl) was mixed with 5 µl of the solution to be tested and incubated at 25° for 15 min, then immediately assayed by ADP-ribosyl transferase activity as described in materials and methods. Incubation was for 5 min.

The enzymatic activity of toxin A and that of the enzymatically active peptide derived from toxin A was heat sensitive (6,16 and Table 11). The enzymatic activity of exoenzyme S was relatively heat stable. As shown in Table 11 at least 90% of the enzymatic activity of either crude or purified toxin A was destroyed by incubation at 100° for 2 min whereas only 30% of the activity of exoenzyme S was lost after 10 min at 100°.

We compared the ability of the toxin A antiserum to neutralize the ADP-ribosyl transferase activity of toxin A and the activity present in the culture supernatant fluid of Ps 388 (toxin S). As shown in Fig. 12, the toxin A antiserum neutralized 80-100% of the enzymatic activity of pure toxin A at dilutions of 1:16 or less. Similar results were obtained using crude toxin A preparations or a purified enzymatically active peptide (14) derived from toxin A. In contrast, even at the highest concentration of toxin A antiserum used (dilution 1:2) the enzymatic activity of toxin S was not decreased (Fig. 12). On the other hand, when rabbit S antiserum is used in place of rabbit A antiserum the opposite is seen. As shown in Table 12, rabbit S antiserum neutralizes the enzymatic activity of toxin S but not that of toxin A. At this time we also tested A antiserum which had been produced in sheep. To our surprise this sheep A antiserum neutralized the enzymatic activity of both A and S (Table 12). However when examined, it was found that the prebled from this sheep (sheep #1) also neutralized S enzymatic activity. Similarly we have found that the sera from a number of sheep contain anti S enzyme neutralizing antibody (Table 12). This anti S activity is purified along with IgG but does not co-purify with specific anti toxin A immunoglobulin that is purified on a toxin A-Sepharose 4B column (Table 12). Anti S activity was also found in 1 sample of normal horse serum, but not in normal pony serum or in anti A toxin serum produced in this pony. Anti S activity was found in only 1 of 4 normal rabbit serum (Table 12). These results indicate that many animals appear to have produced S antibodies which cross react with S whereas we have not found this with toxin A. Thus it is important to test bled animals prior to immunization with toxin A or S. More importantly, we have found it is possible to produce antibodies against S in rabbits and the resultant S antiserum does not cross react with toxin A in the enzyme neutralization test.

Partially purified (stage I) toxin S has been shown under non denaturing and denaturing analytical isoelectric focus gel electrophoresis to have a single peak of enzymatic activity with an isoelectric point between pH 4.4 and pH 4.5. Because of the association of toxin S with other proteins or LPS, at this stage in purification it would be realistic to await further purification before considering these values as accurate. Likewise, SDS gel electrophoresis of stage I DTT treated material demonstrates 2 symetric peaks of enzymatic activity in the presence or absence of reducing agent, a major peak at 60-70 K and a minor peak at 30-35 K daltons.

D. Discussion

These data indicate that toxin S may be produced in medium scale fermentation conditions, but that either it is a minor constituent produced by Ps 388, or proteolysis from proteases during the growth of the organism has contributed

TABLE 11

Comparison of the Thermal Stability of

Pseudomonas Toxin A and Exoenzyme S

	% Unheated Enzymic Activity		
Time (min)	Exoenzyme S	Crude Toxin A	Purified Toxin A
0	100	100	100
2	111	6	10
5	97	3	6
10	71	1	4
20	11	3	2
30	0.3	1	1

Samples (exoenzyme S, 10 µl; crude toxin A, 10 µl, purified toxin A, 0.2 µg) were heated at 100° for the indicated times. After diluting 10 fold in ice-cold 10 mM Tris-HCl (pH 7.4) the samples were assayed for ADP-ribosyl transferase activity as described in Materials and Methods, except that 5 µM [adenine-^{14}C]NAD^{+} (266 mCi/mmole) was used. Incubation was for 5 min.

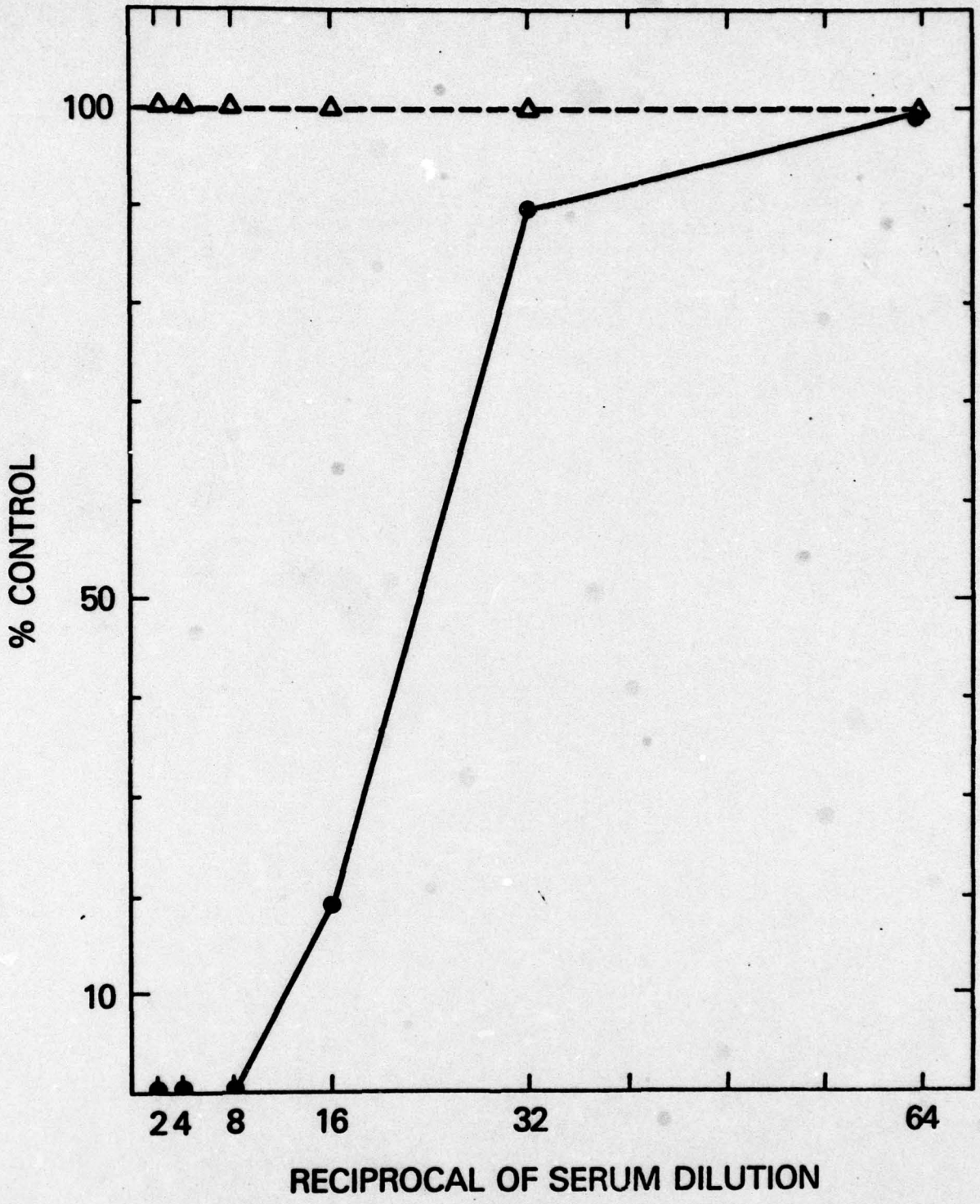


Fig. 12 Neutralization of the enzymatic activity of exoenzyme S and Pseudomonas toxin A by toxin A antiserum. (Δ) exoenzyme S; (o) Pseudomonas toxin A. Inactivation is expressed as the percentage of activity obtained when the toxin or enzyme was preincubated with normal rabbit serum (control).

TABLE 12

Neutralization of Toxin S and Toxin A Enzymatic Activity with Various Antiserum

	% Control	
	Toxin S	Toxin A
NRS (10D)	100%	100%
NRS (11D)	100%	100%
NRS (12A)	68%	100%
NRS (52)	100%	100%
NRS (53)	100%	102%
Rabbit anti S (53F)	5%	100%
Rabbit anti S (52F)	45%	100%
Normal Sheep #1 prebled	9%	100%
Sheep #1 anti A serum	10%	0%
Sheep #1 anti A total IgG	11%	0%
Sheep #1 anti A IgG (toxin A column purified)	100%	0%
Sheep #2 (unimmunized)	16%	100%
Sheep #173	100%	100%
Sheep #181	90%	100%
Sheep #188	47%	100%
Sheep #200	71%	100%
Sheep #282	67%	100%
Normal Horse Serm	58%	100%
Normal Pony Serum #1	100%	100%
Pony Anti A serum	100%	0%

to the complex band patterns (compared to those observed with PA 103) observed on SDS gel electrophoresis. Toxin S has a demonstrated isoelectric point of approximately 4.5 and on SDS gel electrophoresis demonstrates enzymatic activity at approximately 60-70K and 30-35K daltons. Furthermore, the enzymatic activity of exoenzyme S is stabilized by dialysis against DTT and can be potentiated by pretreatment with SDS. These data suggest that, like toxin A, it is possible to find toxin S in a proenzyme and an activated form, with conversion to activated form possibly occurring during some purification step(s). Preparative isoelectric focusing coupled with the previously developed 2 stages of purification promises to yield a convenient purification scheme for obtaining pure toxin S. Neutralizing antibodies have been prepared against an S preparation and the availability of pure S will allow highly specific antibody production to be investigated.

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Pseudomonas aeruginosa exoenzyme S: An adenosine diphosphate–ribosyltransferase distinct from toxin A

(ADP-ribose/NAD$^+$/bacterial exoenzymes/elongation factors 1 and 2/diphtheria toxin)

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ABSTRACT *Pseudomonas aeruginosa* exoenzyme S is an adenosine diphosphate ribosyltransferase distinct from *Pseudomonas* toxin A. Exoenzyme S catalyzes the transfer of radioactivity from all portions of radiolabeled NAD$^+$ except nicotinamide. Digestion of the radiolabeled product(s) formed in the presence of [*adenine*-^{14}C]NAD$^+$ and exoenzyme S with snake venom phosphodiesterase yields only AMP, suggesting that ADP-ribose is present as monomers and not as poly(ADP-ribose). Exoenzyme S does not catalyze the transfer of ADP-ribose from NAD$^+$ to elongation factor 2, as do toxin A and diphtheria toxin, but to one or more other proteins present in crude extracts of wheat germ or rabbit reticulocytes and in partially purified preparations of elongation factor 1. The ADP-ribosyltransferase activity of exoenzyme S is distinct from toxin A by several tests: it is not neutralized by toxin A antibody, it is destroyed rather than potentiated by pretreatment with urea, and it is more heat stable. These latter observations and the substrate specificity suggest that exoenzyme S is different from any previously described prokaryotic ADP-ribosyltransferase.

Diphtheria toxin and *Pseudomonas* toxin A inhibit protein synthesis in eukaryotic cells by catalyzing the transfer of the ADP-ribose (ADP-Rib) moiety of NAD$^+$ to elongation factor 2 (EF-2) (1–3). The only eukaryotic protein known to be modified by these two toxins is EF-2, and all existing information supports the conclusion that the ADP-ribosylation of EF-2 is responsible for the lethality of these two toxins (2, 4). Diphtheria toxin is encoded by a phage gene (2, 5), but the location of the structural gene for *Pseudomonas* toxin A is unknown. Approximately 90% of all isolates of *Pseudomonas aeruginosa* tested produce toxin A (6, 7).

In this report we describe an ADP-ribosyltransferase (exoenzyme S) that is present in the culture supernatant fluid of a strain of *P. aeruginosa* (Ps 388). Ps 388 was consistently negative in an immune precipitation assay (7) using specific toxin A antibody. In the presence of limiting amounts of EF-2, exoenzyme S catalyzed the transfer of far more ADP-Rib from NAD$^+$ than could be accounted for by the production of ADP-ribosylated EF-2. Data are presented to show that exoenzyme S, unlike diphtheria or *Pseudomonas* A toxins, does not modify EF-2 but some other eukaryotic protein(s). ADP-Rib appears to be present in the modified proteins(s) as monomeric units rather than as poly(ADP-Rib). We also show that exoenzyme S is distinct from *Pseudomonas* toxin A by several other tests. It is not neutralized by toxin A antibody, it is destroyed rather than potentiated by pretreatment with urea, and it is more heat stable.

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MATERIALS AND METHODS

Exoenzyme S and Toxins. Ps 388, kindly provided by B. Minshew (Seattle, WA), was grown in a liquid medium adjusted to pH 7.0 consisting of the dialysate from trypticase soy broth (Baltimore Biological Lab.) (8) supplemented with 0.1 M monosodium glutamate, 1% glycerol, and 0.01 M nitrilotriacetic acid (Sigma Chem. Co.). A 25-ml amount of this medium in a 500-ml erlenmeyer flask was inoculated with an overnight culture of Ps 388 to an initial cell density of approximately 5×10^7 cells per ml. The culture was incubated at 32° on a reciprocating shaker (200 linear excursions/min) (Lab.-Line Inst.) for 22 hr. The culture supernatant fluid was obtained by centrifugation at 10,000 × *g* for 20 min at 4°; it was filter sterilized and stored at −70° in small aliquots. Crude exoenzyme S, present in the culture supernatant fluid prepared and stored in this manner, retains enzymatic activity for several months.

P. aeruginosa (PA103) (8) was used as a source of toxin A, which was produced and purified as described (9). The purified toxin A had a mouse median lethal dose (LD_{50}) of 0.2 μg/22-g mouse when injected irtraperitoneally. Fragment A was obtained by treating purified diphtheria toxin with trypsin and dithiothreitol (10). The fragment A was then chromatographed on Sephadex G-100 with 1 mM EDTA/1 mM dithiothreitol/50 mM Tris·HCl, pH 7.8.

Enzymatic Activity. Crude extracts containing aminoacyl transferase factors were prepared from wheat germ as described by Chung and Collier (11). ADP-ribosyltransferase activity was measured by the incorporation of radioactivity from [*adenine*-^{14}C]NAD$^+$ into trichloroacetic acid-precipitable material in the presence of crude wheat germ extracts as previously described (9). Unless otherwise noted, the reaction was performed at 25° for 5 min in 0.1 ml of 50 mM Tris·HCl (pH 7.0), 1 mM EDTA, 50 mM dithiothreitol, 0.12 mM [*adenine*-^{14}C]-NAD$^+$ (10.6 Ci/mol) (Amersham/Searle), wheat germ extract containing 150–160 μg of protein, and various amounts of exoenzyme S, diphtheria toxin fragment A, or *Pseudomonas* toxin A. The reaction was stopped by the addition of 0.1 ml of 10% trichloroacetic acid. The precipitates were collected and washed, and radioactivity was measured as described (9). The enzymatic activity of *Pseudomonas* toxin A was potentiated by first incubating the toxin A in 4 M urea/1% dithiothreitol for 15 min at 25° (12). Where noted, [*nicotinamide*-^{14}C]NAD$^+$ (59 Ci/mol) (Amersham/Searle) or [*Rib*(*NMN*)-^{14}C]NAD$^+$ (75 Ci/mol), prepared as described (13), was substituted for the [*adenine*-^{14}C]NAD$^+$. The following proteins were used in experiments designed to elucidate the substrate specificity of

Abbreviations: ADP-ribose, ADP-Rib; EF, elongation factor.

exoenzyme S: crude extract of rabbit reticulocytes prepared as described by Allen and Schweet (14) as modified by Collier and Kandel (10), purified EF-2 (15) and elongation factor-1 (EF-1) (16) from rabbit reticulocytes (kindly provided by W. Merrick, National Institute of Health), rat liver EF-1 partially purified as described (17), poly(L-lysine) (Sigma), bovine serum albumin (Sigma), egg white lysozyme (Calbiochemicals), and RNase and DNase (Worthington Biochemicals).

Enzyme Neutralization. Specific toxin A antiserum was produced in rabbits as described (7). Enzyme inactivation by antibody was determined by assaying the ADP-ribosyltransferase activity after incubation of 0.1 μg of toxin A or 0.15 μg of crude exoenzyme S with antiserum or normal rabbit serum for 5 min at 37° as described (3). The antiserum was diluted in saline containing 0.1 mg of bovine serum albumin per ml.

Analysis of Reaction Product. Radiolabeled products formed in the ADP-ribosyltransferase assays were electrophoresed in sodium dodecyl sulfate/polyacrylamide gels by a described (12) modification of the method of Weber and Osborn (18). Gel slices (1 mm thick) were incubated 12 hr at 25° in 1 ml of buffer (0.1 M Tris·HCl, pH 7.5/10 mM EDTA/0.1 mg of bovine serum albumin). The eluted protein was precipitated with trichloroacetic acid (5%), collected on Millipore filters, and washed. Radioactivity was measured as described (3).

In order to determine if ADP-Rib was present in the acceptor protein(s) as monomeric units or as poly(ADP-Rib), we incubated a 0.3-ml reaction mixture containing 50 mM Tris·HCl (pH 7.0), 0.03 mM EDTA, 50 mM dithiothreitol, 0.14 mM [*adenine*-^{14}C]NAD$^+$ (26 Ci/mol), rat liver EF-1 (60 μg of protein), and 50 μl of exoenzyme S for 150 min at 25°. At 60, 90, 120, and 150 min, 10-μl aliquots were removed and trichloroacetic acid-precipitable radioactivity was determined. No further incorporation of radioactivity occurred after 120 min. The reaction mixture remaining (260 μl) was chromatographed on a column of Sephadex G-25 (bed volume, 4.0 ml) in 0.2 M Tris·HCl (pH 7.0) to separate the labeled product(s) from unreacted [^{14}C]NAD$^+$. Fractions containing high-molecular-weight radioactive material were pooled (total volume, 0.56 ml) and an aliquot of 250 μl was incubated for 60 min with 240 μg of snake venom phosphodiesterase (Sigma) in a total volume of 0.5 ml containing 0.1 M Tris·HCl (pH 7.0) and 20 mM $MgCl_2$. The products of this digestion were chromatographed on Sephadex G-25 (equilibrated and eluted with water), and the included low-molecular-weight radioactive material was concentrated by lyophilization and resuspended in water. The digested products were then chromatographed on thin-layer polyethyleneimine-cellulose plates (J. T. Baker), with 0.3 M lithium chloride as the solvent (19). AMP and ADP-Rib (Calbiochemicals) and NAD$^+$ (Sigma) were cochromatographed as markers. The chromatogram was cut into small pieces and eluted with 0.3 ml of 1.6 M lithium chloride. After addition of 5 ml of scintillation fluid (16.5 g of diphenyloxazole dissolved in 2 liters of toluene and 1 liter of Triton X-100) radioactivity of the samples was determined in a Nuclear Chicago scintillation counter.

RESULTS

The culture supernatant fluid of *P. aeruginosa* strain 388 (Ps 388) contained activity which, like *Pseudomonas* toxin A (9), transferred radioactivity [*adenine*-^{14}C]NAD$^+$ into acid-insoluble material in the presence of crude wheat germ extracts. Nevertheless, Ps 388 was consistently negative in immune precipitation assays (7) with rabbit toxin A antiserum. Similarly, preparations of culture supernatant fluid from Ps 388 failed to react with toxin A antiserum in agar gel diffusion assays, even when the preparations were concentrated 50-fold and a wide range of antiserum dilutions was tested. We then compared the ability of the toxin A antiserum to neutralize the ADP-ribosyltransferase activity of toxin A and the activity present in the culture supernatant fluid of Ps 388 (exoenzyme S). As shown in Fig. 1, the toxin A antiserum neutralized 80–100% of the enzymatic activity of pure toxin A at dilutions of 1:16 or less. Similar results were obtained with crude toxin A preparations or a purified enzymatically active peptide (12) derived from toxin A (data not shown). In contrast, even at the highest concentration of toxin A antiserum used (dilution 1:2), the enzymatic activity of exoenzyme S was not decreased (Fig. 1).

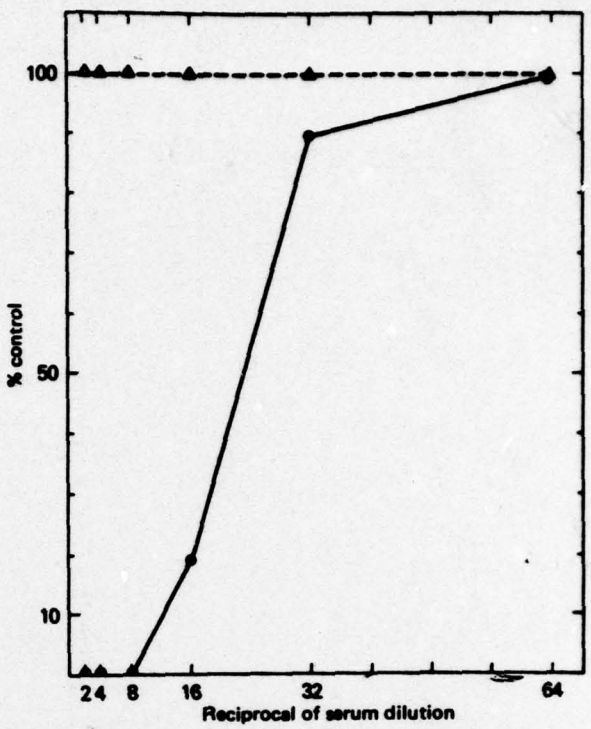

FIG. 1. Neutralization of the enzymatic activity of exoenzyme S and *Pseudomonas* toxin A by toxin A antiserum. △, Exoenzyme S; ●, *Pseudomonas* toxin A. Inactivation is expressed as the percentage of activity obtained when the toxin or enzyme was incubated with normal rabbit serum (control).

To determine which portions of NAD$^+$ are transferred by exoenzyme S, we incubated the enzyme with wheat germ extracts and NAD$^+$ radioactively labeled at different positions. All portions of NAD$^+$ except nicotinamide were incorporated into trichloroacetic acid-insoluble reaction product(s) in the presence of exoenzyme S or toxin A (Table 1). Thus, exoenzyme S is an ADP-ribosyltransferase.

Pseudomonas toxin A is produced as a toxic proenzyme that is virtually devoid of ADP-ribosyltransferase activity (12, 20). The transferase activity of toxin A is expressed when the molecule is cleaved by proteolysis to yield an enzymatically active fragment (11, 12) or when it is denatured and reduced (12, 20). In contrast, we found that treatment of exoenzyme S with 4 M urea markedly reduced its enzymatic activity. The presence of dithiothreitol slightly modified the effect of urea on exoenzyme S activity (Table 2).

The enzymatic activity of toxin A and that of the enzymatically active peptide derived from toxin A was heat sensitive (refs. 3 and 11 and Table 3). The enzymatic activity of exoenzyme S was relatively heat stable. As shown in Table 3, at least

Table 1. Incorporation of label from NAD^+ preparations into protein by exoenzyme S

NAD^+ used	Radioactivity incorporated, cpm	
	No enzyme	Enzyme
[*Adenine*-^{14}C]NAD^+	180	26,000
[*Rib(NMN)*-^{14}C]NAD^+	220	3,560
[*Nicotinamide*-^{14}C]NAD^+	110	118

[*Adenine*-^{14}C]NAD, 5.0 μM (266 Ci/mol), [*Rib(NMN)*-^{14}C]NAD^+, 3.0 μM (75 Ci/mol), or [*nicotinamide*-^{14}C]NAD^+, 5.0 μM (59 Ci/mol) was added to the reaction mixtures containing 10 μl of H_2O or 10 μl of exoenzyme S, 150 μg of wheat germ extract, and reaction buffer.

90% of the enzymatic activity of either crude or purified toxin A was destroyed by incubation at 100° for 2 min, whereas only 30% of the activity of exoenzyme S was lost after 10 min at 100°. The data in Fig. 1 and Tables 2 and 3 suggest that exoenzyme S and toxin A are structurally different.

The data in Table 4 show that in the presence of excess NAD^+ and limiting amounts of wheat germ extract, exoenzyme S catalyzed the transfer of far more ADP-Rib than could be accounted for by the formation of an ADP-ribosylated EF-2 product such as has been described (1–4). When excess toxin A or fragment A was incubated with limiting amounts of wheat germ extract and excess NAD^+, approximately 32 pmol of ADP-Rib was transferred from [*adenine*-^{14}C]NAD^+ to acid-insoluble material. Doubling the amount of toxin A or fragment A did not increase the amount of ADP-Rib transferred. Under the same conditions, 10 μl of exoenzyme S catalyzed the transfer of 3670 pmol of ADP-Rib.

An investigation of the product formed in the presence of exoenzyme S revealed it to be stable to treatment with DNase or RNase but digestible with trypsin or Pronase. When this radiolabeled product was subjected to electrophoresis on sodium dodecyl sulfate/polyacrylamide gels, the acid-precipitable material obtained after elution of the individual gel slices indicated that the product was heterogeneous in size (Fig. 2). No significant amount of radioactivity was associated with material larger than 50,000 daltons. In agreement with previous reports (3, 4), the product formed in the presence of toxin A was homogeneous and had a molecular weight of 100,000 (the known molecular weight of EF-2) (Fig. 2).

These data (Table 4 and Fig. 2) suggested that EF-2 was not the acceptor for ADP-Rib in the reaction catalyzed by exoenzyme S. This was confirmed in experiments with highly purified reticulocyte EF-2. As shown in Table 5, exoenzyme S failed to transfer [*adenine*-^{14}C]ADP-Rib from [*adenine*-^{14}C]NAD^+ to pure EF-2. Under the same conditions, 0.2 μg of toxin A catalyzed the transfer of 2050 pmol of ADP-Rib to this preparation of EF-2 (data not shown).

Table 2. Effect of urea and dithiothreitol on ADP-ribosyltransferase activity of exoenzyme S and *Pseudomonas* toxin A

Treatment	ADP-Rib incorporated, pmol	
	Toxin A	Exoenzyme S
H_2O	0.72	1200
1% Dithiothreitol	0.67	1250
4 M Urea	10.9	760
4 M Urea + dithiothreitol	26.1	960

Pseudomonas toxin A (0.02 μg) or exoenzyme S (5 μl) was mixed with 5 μl of the solution to be tested and incubated at 25° for 15 min, then immediately assayed for ADP-ribosyltransferase activity.

Table 3. Comparison of thermal stability of *Pseudomonas* toxin A and exoenzyme S

Time, min	% unheated enzymic activity		
	Exoenzyme S	Crude toxin A	Purified toxin A
0	100	100	100
2	111	6	10
5	97	3	6
10	71	1	4
20	11	3	2
30	0.3	1	1

Samples (exoenzyme S, 10 μl; crude toxin A, 10 μl; purified toxin A, 0.2 μg) were heated at 100° for the indicated times. After the samples were diluted 1:10 in ice-cold mM Tris-HCl (pH 7.4), they were assayed for ADP-ribosyltransferase activity as described in *Materials and Methods*, except that 5 μM [*adenine*-^{14}C]NAD^+ (266 Ci/mol) was used.

Various other proteins were tested for acceptor activity in the reaction catalyzed by exoenzyme S (Table 5). Poly(L-lysine), bovine serum albumin, egg white lysozyme, and RNase were inactive. The two EF-1 preparations tested contained acceptor activity. The rat liver EF-1 and reticulocyte EF-1 preparations, purified by different methods, were reported to be 50 and 95% pure, respectively (16, 17). The acceptor(s) could well be contaminating proteins and not EF-1. However, a substantial amount of the radioactive product in wheat germ extract was associated with material of about 50,000 molecular weight (Fig. 2), a result consistent with ADP-ribosylation of EF-1. The smaller labeled material (Fig. 2) might be the result of proteolysis since the exoenzyme S preparation contains contaminating proteolytic enzymes.

Previously described prokaryotic ADP-ribosyltransferases have been shown to transfer single molecules of ADP-Rib from NAD^+ directly to proteins and not to each other in a repetitive fashion to yield poly(ADP-Rib) (21). In order to determine if the protein(s) modified by exoenzyme S contained monomers or polymers of ADP-Rib, we synthesized the product(s) formed in the presence of exoenzyme S, [*adenine*-^{14}C]NAD^+, and rat liver EF-1 and treated then as described in *Materials and Methods*. Eighty-two percent (2.0 nmol) of the acid-precipitable radioactivity in the original reaction mixture was recovered from Sephadex G-25 as high-molecular-weight material. After incubation with venom phosphodiesterase only 62% of the applied radioactive material was recovered, but all the recovered activity was in low-molecular-weight fractions. Ninety-three percent of this low-molecular-weight material

Table 4. Comparison of ADP-ribosyltransferase activity of exoenzyme S, *Pseudomonas* toxin A, and diphtheria toxin fragment A

Enzyme	ADP-Rib incorporated, pmol
Diphtheria toxin fragment A: 0.1 μg	29
0.2 μg	33
Pseudomonas toxin A: 0.1 μg	33
0.2 μg	32
Exoenzyme S: 10 μl	3670

Activity was determined as described in *Materials and Methods* with incubation for 15 min.

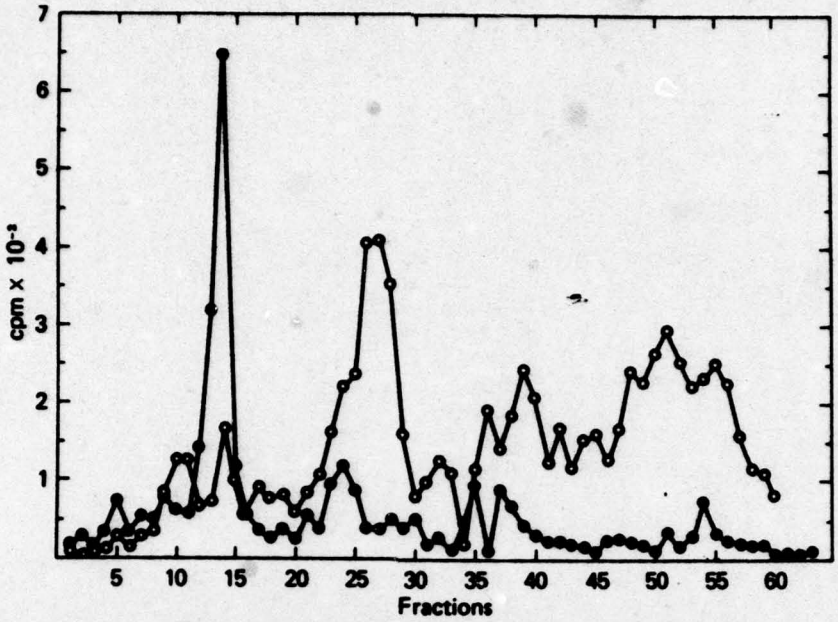

FIG. 2. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of acid-precipitable radiolabeled products formed in the presence of wheat germ extracts, 0.5 μg of *Pseudomonas* toxin A (●) or 1.0 μg of crude exoenzyme S (○), and 5 μM [*adenine*-^{14}C]NAD$^+$ (266 Ci/mol). Incubation was for 15 min.

chromatographed with AMP on polyethyleneimine-cellulose plates. No radioactivity migrated with marker ADP-Rib [which comigrates in the system used with *iso*ADP-Rib, the product of venom phosphodiesterase action on poly(ADP-Rib)] (21). When the product formed with wheat germ extract was analyzed similarly, the only radioactive product recovered after snake venom phosphodiesterase digestion was AMP. This strongly suggests that ADP-Rib is present on the acceptor(s) in both wheat germ extracts and in the rat liver EF-1 preparation as monomeric units, rather than as poly(ADP-Rib).

DISCUSSION

Five prokaryotic ADP-ribosyltransferases have been described: diphtheria toxin (1, 2), *Pseudomonas* toxin A (3, 4), T4 phage "alteration" enzyme (22), T4 phage "modification" enzyme (23), and an N4 phage enzyme (24). Existing evidence indicates that these enzymes play major roles in infection (i.e., diphtheria toxin) or in the regulation of various cellular activities (i.e., T4 modification enzyme) (2, 21). In addition, preliminary evidence has been presented that choleragen may be an ADP-ribosyltransferase (25).

Table 5. Incorporation of [*adenine*-^{14}C]ADP-Rib into acid-insoluble material with exoenzyme S and various substrates

Substrate	ADP-Rib incorporated, pmol
Wheat germ extract (160 μg)	3890
Reticulocyte extract (150 μg)	358
Reticulocyte EF-2 (76 μg)	0
Poly(L-lysine)(50 μg)	0
Bovine serum albumin (50 μg)	0
Lysozyme (50 μg)	0
RNase (85 μg)	0
Rat liver EF-1 (13 μg)	220
Reticulocyte EF-1 (20 μg)	45
H_2O (25 μl)	0

The reaction was carried out as described in *Materials and Methods* with 10 μl of exoenzyme S and an incubation time of 15 min, with the following exceptions: both preparations of EF-1 were incubated for 60 min and the NAD$^+$ concentration was 0.14 mM (16.5 Ci/mol).

The present results demonstrate the existence of a sixth prokaryotic ADP-ribosyltransferase, exoenzyme S, which is produced by some strains of *P. aeruginosa*. Exoenzyme S differs immunologically from *Pseudomonas* toxin A, is heat stable relative to toxin A, and is partially inactivated by conditions that potentiated the enzymatic activity of toxin A (Fig. 1 and Tables 2 and 3). Exoenzyme S catalyzed the transfer of ADP-Rib from NAD$^+$ to protein(s) present in wheat germ extracts, rabbit reticulocyte extracts, and partially purified EF-1 preparations (Tables 4 and 5). EF-2 failed to serve as an acceptor in the reaction catalyzed by exoenzyme S, indicating a substrate specificity different from that of *Pseudomonas* toxin A and diphtheria toxin. Furthermore, lysozyme, which serves as an acceptor in the *in vitro* reaction catalyzed by the T4 phage alteration enzyme (22) and the N4 virion-associated enzyme (24), was not ADP-ribosylated in the presence of exoenzyme S. The T4 modification enzyme reportedly does not ADP-ribosylate proteins present in rabbit reticulocyte extracts (2) as did exoenzyme S (Table 5). Thus, exoenzyme S differs in its substrate specificity from previously described prokaryotic ADP-ribosyltransferases.

Our results raised the possibility that exoenzyme S ADP-ribosylates EF-1. As seen in Table 5, in the presence of exoenzyme S [^{14}C]ADP-Rib was transferred from [*adenine*-^{14}C]-NAD$^+$ to trichloroacetic acid-insoluble material when EF-1 preparations were used as the substrate. The rat liver EF-1 preparation was approximately 50% pure and contained four or five proteins other than EF-1 (17). The reticulocyte EF-1 preparation was approximately 95% pure (16). These proteins were purified by different procedures (16, 17). Considerably more ADP-Rib per μg of protein was transferred to the rat liver preparation than to the reticulocyte preparation. Thus, if exoenzyme S is ADP-ribosylating EF-1, then either EF-1 from different sources differs in the extent to which it can be modi-

fied by exoenzyme S or it may somehow be altered during purification. A third, perhaps more likely, possibility is that some other protein(s) contaminating the EF-1 preparations is being ADP-ribosylated by exoenzyme S and this protein is in a smaller concentration in the more highly purified reticulocyte EF-1 preparation than in the rat liver EF-1 preparation. Purification of exoenzyme S to remove proteolytic contaminants, together with the use of recently developed methods for purifying catalytically active EF-1 and EF-1 α and β factors (26), should provide the necessary reagents to distinguish among these possibilities.

The role of diphtheria toxin in *Corynebacterium diphtheriae* infections is clear (2). There is increasing evidence that toxin A is also important in the pathogenesis of disease caused by *P. aeruginosa* (6, 7, 27). *P. aeruginosa* strain 388 produces exoenzyme S but no detectable amounts of toxin A. More recently we have identified other strains of *P. aeruginosa* that produce exoenzyme S and several that produce both toxin A and exoenzyme S.

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